B cell targeting in Sjögren's syndrome: clues to pathogenesis

Rodney Pollard

B CELL TARGETING IN SJÖGREN'S SYNDROME

CLUES TO PATHOGENESIS

Thesis

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Awinsi mi e taki san gado gi mi fu taki,
èn mi sabi ala kibritori nanga ala sani di
de fu sabi, awinsi mi abi nofo bribi fu puru
wan bergi na en presi, ma mi no abi
lobi, dan mi no de noti.

I Korinte 13:2 (sranan tongo)

Contents

CHAPTER 1

Introduction and aims of the study

Introduction

Sjögren's syndrome (SS) is a complex systemic autoimmune disease hallmarked by chronic inflammation of exocrine glands, in particular lacrimal and salivary glands. In these glands lymphocytic infiltrates lead to destruction of the excretory tissue resulting in dry eyes (keratoconjunctivitis sicca) and dry mouth (xerostomia) [1]. Although keratoconjunctivitis sicca and xerostomia are the typical symptoms of SS, the disease may affect almost all organ systems of the body. Extraglandular manifestations may involve skin, joints, lungs, liver, kidneys, thyroid gland, lymph nodes, blood cells, central nervous system, vagina and skin, and may cause substantial morbidity to the affected individual and interfere with quality of daily life [1-3].
SS is one of the most prevalent autoimmune diseases with an estimated prevalence of 1 to 3% and with a female over male preponderance of 9 : 1 [4, 5]. SS may occur alone as primary SS (pSS) or as secondary SS (sSS) in conjunction with other autoimmune disease such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) [1, 6, 7].

Brief historical overview

In 1888, the surgeon Johan Mikulicz (1850-1905) described a case of painless bilateral swelling of the lacrimal, parotid and submandibular glands. Surgical specimens of the lacrimal and both submaxillar glands contained numerous small round cells suggestive of lymphoma (figure 1) [8]. In 1933, during his training of ophthalmology, Henrik Sjögren (1899-1987) described a variety of clinical and histopathological findings in patients with symptoms of RA, dry eyes and dry mouth. He introduced the term "keratoconjunctivitis sicca" and was the first to appreciate the systemic nature of SS [9].

The relationship between the case described by Johan Mikulicz and the cases of SS described by Henrik Sjögren was clarified by Morgan et al. [10] in 1954 who demonstrated that the histologic infiltrate in the salivary gland tissue showed a remarkable resemblance between the Mikulicz case and the SS cases described by Henrik Sjögren, stating that these cases reflect probably the same entity. Ihrler et al. analysed the Mikulicz case of 1888 and identified a mucosa associated lymphoid tissue (MALT)-lymphoma [11]. Indeed subsequent studies confirmed the relationship between SS and development of lymphoma [12, 13].

Diagnosis

SS is characterized by a variety of presenting symptoms that may either be rather specific for SS or may arise in other autoimmune and related (e.g., HIV, sarcoidosis) disorders as well. Thus, over time there have been a number of classification criteria sets how to classify a patient as SS. In 2002, an international group reached consensus

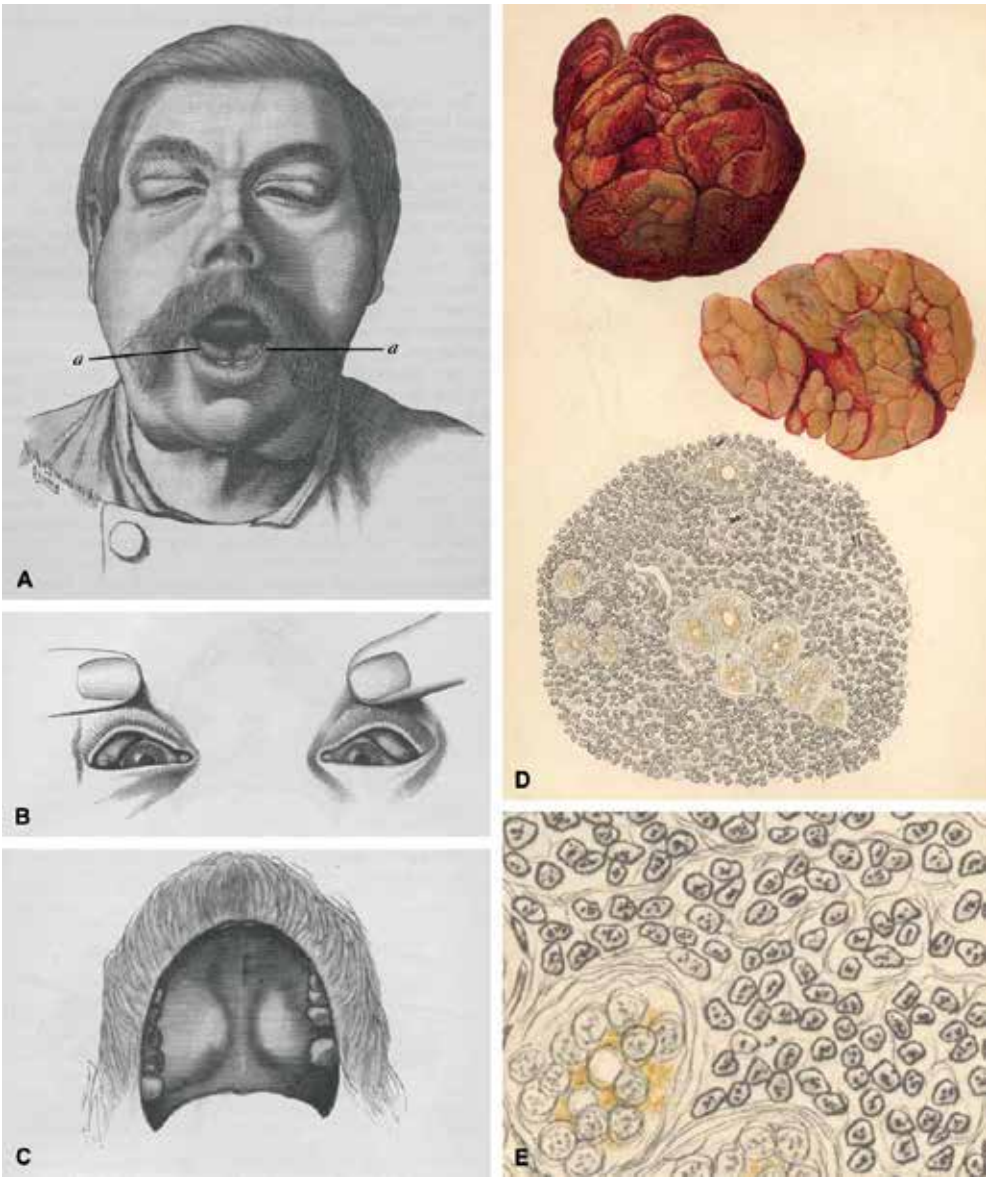

Figure 1. Panels A-C show the woodcuts that appeared in the text of Mikulicz's original article. A, Extensive symmetrical swelling of the lacrymal, parotid, submandibular, and sublingual (labelled a) glands. B, Manual lifting of the eyelids highlights the extensive swelling of the lacrymal glands. C, Extensive symmetrical swelling of the palatal minor salivary glands. Panel D is the original color print that appeared with the article. This panel consists of macroscopic and histologic drawings of the left submandibular gland. The external appearance (top) and the transverse section (middle) show an enlarged gland with a preserved lobular structure. The microscopic field (bottom) shows a uniform cellular infiltrate with 3 mitoses and scattered serous acini with orange secretory granules. Panel E shows the central part of the microscopical field of the original color print magnified to 240% revealing the nuclei of the cells of the infiltrate to be smaller than the nuclei of the acini. These small to medium-sized nuclei of the cells of the infiltrate are irregularly shaped and contain dispersed chromatin without obvious nucleoli. Although the cytoplasm of the infiltrate is unstained, the separation between the nuclei indicates that it is abundant. These features are those of lymphocytes of a centrocyte-like morphology (reprinted with permission from prof. dr. S. Ihrler [11])

Table 1. The 2002 American-European classification criteria for SS [14].

I. Ocular symptoms: a positive response to at least one of the following questions: 1. Have you had daily, persistent, troublesome dry eyes for more than 3 months? 2. Do you have a recurrent sensation of sand or gravel in the eyes? 3. Do you use tear substitutes more than 3 times a day?
II. Oral symptoms: a positive response to at least one of the following questions: 1. Have you had a daily feeling of dry mouth for more than 3 months? 2 Have you had recurrently or persistently swollen salivary glands as an adult? 3. Do you frequently drink liquids to aid in swallowing dry food?
III. Ocular signs- that is, objective evidence of ocular involvement defined as a positive result for at least one of the following tests: 1. Schirmer's test, performed without anaesthesia (<5mm in 5 minutes) 2. Rose bengal score or other ocular dye score (>4 according to van Bijsterveld's scoring system)
IV. Histopathology: in minor salivary glands (obtained through normal-appearing mucosa) focal lymphocytic sialoadenitis, evaluated by an expert histopathologist, with a focus score ≥1, defined as a number of lymphocytic foci (which are adjacent to normal-appearing mucous acini and contain more than 50 lymphocytes) per 4 mm2 of glandular tissue.
V. Salivary gland involvement: objective evidence of salivary gland involvement defined by a positive result for at least one of the following diagnostic tests: 1. Unstimulated whole salivary flow (<1.5 ml in 15 minutes) 2. Parotid sialography showing the presence of diffuse sialectasias (punctuate, cavitary or destructive pattern), wihtoud evidence of obstruction in the major ducts 3. Salivary scintigraphy showing delayed uptake, reduced concentration and/or delayed excretion of tracer
VI. Autoantibodies: presence in the serum for autoantibodies to Ro(SSA) or La(SSB) antigens, or both

For primary SS: in patients without any potentially associated disease, primary SS may be defined as follows:

a. The presence of any 4 of the 6 items is indicative of primary SS, as long as either item IV (Histopathology) or VI (Serology) is positive **or**
b. The presence of any 3 of the 4 objective criteria items (that is, items III (Ocular signs), IV (Histopathology), V (Salivary gland involvement), or VI (Serology)).

For secondary SS: in patients with a potentially associated disease (for instance, another well defined conncective tissue disease), the presence of item I or item II plus any 2 from among items III, IV and V may be considered as indicative of secondary SS.

Exclusion criteria: past head and neck radiation treatment, hepatitis C infection, acquired immunodeficiency disease (AIDS), pre-existing lymphoma, sarcoidosis, graft versus host disease, use of anticholinergic drugs (since a time shorter than 4-fold the half life of the drug)

on a set of American/European criteria for SS classification (table 1) [14]; this criteria set is currently the most commonly applied criteria set worldwide. These international American/European Consensus Group classification (AECG) criteria comprise subjective (ocular and oral symptoms) and objective criteria (ocular, histopathological, oral and serological signs) [1, 14, 15]. It must be noted that the AECG criteria for SS were not developed for clinical purposes but to select cohorts of SS patients for performing clinical trials with well-defined SS subjects, e.g., for assessing the effect of a particular therapy and for allowing reliable comparison of results of various published studies. Nonetheless the AECG criteria are now worldwide accepted as diagnostic tools for SS, but modifications to these criteria have been suggested as the AECG

Table 2. The 2012 American College of Rheumatology criteria for SS [16].

The classification of SS will be met in patients who have at least 2 of the following 3 objective features:

I. Positive serum anti-SSA/Ro and/or anti-SSB/La or (positive rheumatoid factor and ANA titer 1:320)
II. Labial salivary gland biopsy exhibiting focal lymphocytic sialadenitis with a focus score ≥1 focus/4 mm^2
III. Keratoconjunctivitis sicca with ocular staining score ≥3 (assuming that individual is not currently using daily eye drops for glaucoma and has not had corneal surgery or cosmetic eyelid surgery in the last 5 years)

Prior diagnosis of any of the following conditions would exclude participation in SS studies or therapeutic trials because of overlapping clinical features or interference with criteria tests: history of head and neck radiation treatment, hepatitis C infection, acquired immunodeficiency syndrome (AIDS), sarcoidosis, amyloidosis, graft versus host disease, IgG4-related disease.

criteria comprise subjective signs and certain salivary and ophthalmologic criteria lack specificity [16-18]. In 2012, the American College of Rheumatology (ACR) introduced a provisional criteria set (table 2) [16]. The ACR criteria were established, in part, to improve the specificity of criteria used for entry of SS patients into clinical trials [16]. These recent ACR criteria differ from the AECG criteria by excluding criteria based upon symptoms of glandular manifestations (i.e. xerostomia and keratoconjunctivitis sicca) and not distinguishing between pSS and sSS. Labial salivary gland biopsies and serology, however, remain the main criteria in both criteria sets.

Although labial salivary gland biopsies are a main criterion in both the AECG and ACR criteria sets, studies comparing labial and parotid gland biopsies have shown that incisional parotid gland biopsies were superior to or at least comparable with labial biopsies in the diagnostic workup of SS according to the AECG criteria [15, 19, 20]. Parotid gland biopsies have advantages over labial biopsies. In both biopsy techniques the morbidity is low, but permanent skin hypoesthesia mainly occurs after a labial gland biopsy. Another main advantage of parotid gland biopsies relies on the fact that the parotid gland can be biopsied more often. Parotid biopsies are, therefore, not only a valuable diagnostic asset but also fundamental in monitoring disease treatment at the glandular level. With regard to the ACR criteria, the use of the parotid gland biopsy as a replacement for the labial salivary gland biopsy has still to be validated.

In the various studies described in this thesis emphasis is put on what can be learned from parotid gland biopsies, both regarding the etiopathogenesis of SS as well as the effect of treatment of SS with a biological on a tissue level.

Pathogenesis

The etiology of SS remains undefined, although it is believed that the pathogenesis of exocrine cell damage is multifactorial, involving viral, genetic, hormonal and immunological components.

Several studies have focused on the etiopathogenic role of viruses in SS [21-24]. Despite many clinical and experimental studies that are carried out, no evidence for

a direct pathogenic role of viruses in SS has yet been demonstrated. It remains to be determined whether viral infection of the affected glands is primary or secondary to the development of autoimmunity in pSS (for example Epstein–Barr virus infection [25]) or whether certain viruses, such as human T lymphotropic virus type I [26] and coxsackievirus [27], may act as triggers in distinct human populations for the development of SS.

A genetic predisposition to SS is probably present since several families involving two or more cases of SS have been described including monozygotic twin studies [28-31]. Predisposition to pSS in caucasians is associated with an increased frequency of the HLA-DR3 gene [32, 33]. HLA-DR3 appears to be more closely associated with autoantibody production, exemplified by the notion that patients producing anti-Ro/SSA or anti-La/SSB autoantibodies are associated with a frequency of 60 to 90 percent in HLA-DR3 positive patients [34]. The level of genetic contribution is not yet known.

The strong female predominance and the menopausal age of diagnosis suggest sex-specific predisposing factors [35, 36]. A mouse model of estrogen deficiency resulted in a SS like disease with increased apoptosis in salivary and lacrimal glands, suggesting a role in the development of autoantigens [37].

Immunologic components are involved in the pathogenesis of SS. In blood, immunologic activity can be detected ranging from hypergammaglobulinemias, high erythrocyte sedimentation rate and C-protein levels, and autoantibodies [38, 39].

Regarding the salivary glands, former studies have shown that minor salivary glands become infiltrated with T lymphocytes, although substantial numbers of B cells and plasma cells are also present in the inflamed excretory tissue together with (plasmacytoid) dendritic cells and macrophages [40-42]. Characteristically, the salivary glands are infiltrated around the striated ducts by T- and B lymphocytes [43]. The epithelial cells of striated ducts are a likely target of the autoimmune attack. Aberrant apoptosis of epithelial cells has been proposed to play a critical role in the pathogenesis of SS. Pro-inflammatory cytokines and chemokines are driving forces of migration of immune cells to salivary and lacrimal glands [44-47]. Elevated levels of these chemokines and cytokines are found in the glandular tissue, saliva, tears and serum of pSS patients [44-47]. The ductal epithelium appears to be an important source of these cytokines and chemokines. Thus the ducts are not only target of the autoimmune process but also serve important immunological functions, central in the pathogenetic process. Within the glandular tissue, these infiltrates can even be organized to ectopic lymphoid tissues with segregated T- and B-cell areas, germinal center like structures and high endothelial venules [48-50]. Th17 cells are believed to play an important role in the initiation of tissue inflammation, the infiltration of other inflammatory cells and the formation of ectopic lymphoid tissue. The ectopic lymphoid tissue contains all elements for local, auto-antigen driven cellular and humoral immune responses.

The clinical manifestations of SS, such as hyposalivation, reduced tear secretion and autoantibody production, have been suggested to be mediated through B cell differentiation, activation, cytokine production and altered B cell tolerance [1, 51-53]. B cell hyperreactivity plays a crucial role in the development and perpetuation of pSS disease. Autoantibodies directed against Ro/SSA and La/SSB antigens and/or rheumatoid factor are frequently found in pSS patients, together with elevated levels of serum immunoglobulins (hyper-gammaglobulinemia) [54].

Although autoantibodies are frequently found in pSS patients, the pathogenetic role for these autoantibodies remains unclear [55, 56]. At least part of the autoantibodies might be generated in the exocrine tissues from local immune responses. Indeed autoantibody forming cells have been found in salivary glands of pSS patients [57]. Furthermore, anti-SSA/SSB antibodies are present in saliva and tears [58, 59]. Effector CD4+ Th cells are required for humoral immune responses to these protein autoantigens. These CD4+ Th cells are essential for the generation of plasma cells, as well as for the formation of memory cells in the germinal center like structures in the salivary glands [60]. CD4+ Th cells are also critically involved in the inflammatory response in the exocrine glands and their cytokines affect directly the ductal epithelial cells [40].

B cell activating factor (BAFF) is a B cell survival factor required for B cell maturation and known to regulate B lymphocyte proliferation and survival. Excess production of BAFF in BAFF- transgenic (Tg) mice leads to autoreactive B cell formation [61]. It was demonstrated that BAFF Tg mice initially develop an autoimmune condition similar to SLE and eventually develop sSS that manifests as sialadenitis and destruction of salivary glands when the mice mature [61]. Moreover, BAFF seems to be involved in the formation of ectopic germinal centers (GCs) in the salivary glands which may be an important step in lymphomagenesis. Similar to pSS, ectopic lymphoid tissue is also present in target tissues of several other autoimmune and non-autoimmune conditions that are accompanied by B cell disturbances and/or enhanced lymphoma risk, for example RA [62], SLE [63], autoimmune thyroiditis [64] and chronic infections such as human immunodeficiency virus (HIV) [65] and hepatitis C virus [66]. Plasma cells secreting (auto-) antibodies are derived from activated B cells undergoing GC reaction [67, 68]. Together with the fact that both Ro/SSA and La/SSB antigens have been detected in the salivary glands of SS patients, it is plausible that autoimmune plasma cells are produced at the site of inflammation [69, 70]. Furthermore, the pSS salivary gland microenvironment provides niches rich in factors, such as CXCL12 and IL-6, vital for plasma cell survival [71]. No studies yet exist in which the role of B cells and B cell mediated factors are evaluated after depletion of these B cells.

Chronic inflammation with ectopic lymphoid tissue appears to induce neoplastic transformation of lymphocytes from ectopic GCs, leading to MALT lymphoma [72]. Salivary gland MALT lymphomas are usually preceded by SS-derived lymphoepithelial

lesions [73] and are mostly composed by CD20 positive B cells [74]. Taken together, these reports illustrate an important interplay between B cells, cytokines, the GC microenvironment and salivary gland tissue in the perpetuation and progression of SS. However, no reports exist where these factors have been studied simultaneously in a human SS disease state before initiation and/or progression, and when clinical symptoms reappear after treatment with biologicals.

B cell targeted therapy

Rituximab, a chimeric monoclonal antibody to the CD20 antigen, is a biological that has been used with success in the treatment of B cell lymphoma [75]. B cell targeting by rituximab has also been beneficial in autoimmune diseases like RA [76], SLE [77], multiple sclerosis [78] and myositis [79]. B cells appear to be critical in the pathogenesis of pSS too as exemplified by production of autoantibodies and cytokines. Therefore, although the direct pathophysiological role of B cells in glandular tissue destruction has not been fully elucidated, B cell targeted treatment has been proposed as a therapeutic modality in pSS [80]. Several open label and small placebo-controlled randomized clinical trials with rituximab treatment have been conducted demonstrating the potential beneficial clinical effects of B cell targeting in pSS, such as improvement in extraglandular manifestations and diminished fatigue [81-84]. The beneficial clinical effects of rituximab seem to last for 6-9 months after treatment [83]. The direct effects of rituximab on systemic and local immunopathology remain, however, to be elucidated. The depletion and subsequent reappearance of B cells in conjunction with a return of clinical symptoms provide a unique opportunity to study immunologic changes that may precede or accompany the return of disease activity in SS. Such data might provide clues to the pathogenesis of SS.

Aim and outline of the thesis

The main objective of this thesis was to assess the effect of B cell targeting by rituximab on systemic and local (i.e. parotid salivary gland tissue) immunopathology in pSS. In addition, it was assessed, by comparing the results of a system analysis of salivary gland tissue obtained from human and mice with SS, which factors may be involved in the pathogenesis of pSS. Finally, an algorithm was developed how to deal with SS patients with a mucosa associated tissue B cell lymphoma (MALT). MALT lymphomas pose a problem in SS patients as about 7.5% of SS patients develop such a lymphoma [7, 13]. Besides in treating SS, rituximab has also been used in the treatment of MALT lymphomas. In short:

pSS mouse studies have hinted to factors (a.o. cytokines and lymphocytes) that might be involved in the pathogenesis of human SS. The exact role of these factors

in human pSS pathogenesis remains obscure as the intersection between pSS mouse models and human pSS has not been properly studied. In **chapter 2** weighted gene co-expression network analysis (WGCNA) is applied to characterize molecular and cellular events associated with pSS pathogenesis. Factors involved in the pathogenesis of human pSS might resemble factors involved in the pathogenesis of SS-like disease in mouse models. Aim of the study described in this chapter was to assess whether WGCNA identifies corresponding biological pathways and targets in pSS pathogenesis between human with SS and mice with a SS-like disease. Factors studied in the subsequent chapters were selected on basis of and/or linked to the results of the study described in this chapter.

In **chapter 3** the results of a randomized placebo controlled clinical trial assessing the effect of rituximab on the histology of excretory parotid salivary gland tissue in patients with pSS are discussed. In this prospective study, 20 patients with pSS had been treated with rituximab and 10 with placebo. Parotid gland histology was evaluated at baseline and at 12 weeks after rituximab treatment. Emphasis was put on the effect of rituximab on the local inflammation and the possible regenerative potential of parotid salivary gland tissue.

The observations in chapter 3 were on the basis of the study described in **chapter 4**, where the effect of rituximab was evaluated on the circulating and parotid tissue bound levels of BAFF and a proliferation-inducing ligand (APRIL). These cytokines are important regarding B cell survival and activation and have been shown to be elevated in pSS patients [85].

Cytokines and chemokines are thought to play a critical role in the initiation and perpetuation of the chronic inflammatory response in the glandular tissue of patients with pSS [86, 87]. Several pro-inflammatory cytokines have shown to be overexpressed in saliva and serum of pSS patients [88-90] as well as that B cells are able to produce cytokines [91]. The effect of rituximab on a variety of cytokines was therefore investigated in **chapter 5**, with notice on the assumption that the therapeutic effect of rituximab on disease activity in pSS patients is mediated by a depletion of cytokine producing B cells.

Chapters 4 and 5 showed effect of rituximab on serum levels of cytokines and chemokines in pSS patients. Cytokines and chemokines play a crucial role in T cell activation, but the effect of rituximab on T cells is unclear. T regulatory (Treg) are presumed to suppress activation of the immune system and to prevent pathological self-reactivity, i.e. autoimmunity. The study in **chapter 6** was performed to assess the

effect of rituximab on T regulatory (Treg) cells in pSS, both locally in inflamed parotid gland tissue and systemically in blood.

Abnormal B cell stimulation by immune factors, as assessed in the studies described in chapters 3 to 6, may eventually result in B cell lymphoproliferation and give rise to development of MALT-lymphoma. SS related MALT-lymphoma ranges from indolent asymptomatic lymphoma with no pSS disease activity to disseminated lymphoma with severe extraglandular pSS manifestations. As no clear guidelines are available for the management of pSS patients with a MALT-lymphoma, the progression and treatment of a MALT-lymphoma in 35 consecutive pSS patients with such a lymphoma was studied and linked to their clinical characteristics, the course of the disease and their response to a variety of treatment strategies. In **chapter 7** guidelines for the treatment of MALT-lymphoma in patients with pSS are given which are based on these data.

In **chapter 8**, the findings of this thesis are summarized and discussed, and perspectives for future studies are given.

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CHAPTER 1

CHAPTER 2

Systems analysis of primary Sjögren's syndrome pathogenesis in salivary glands identifies shared pathways in human and a mouse model

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Abstract

Objective

Primary Sjögren's syndrome (pSS) is a chronic autoimmune disease with complex etiopathogenesis. Despite extensive studies to understand the disease process utilizing human and mouse models, the intersection between these species remain elusive. To address this gap, we utilized a novel systems biology approach to identify disease-related gene modules and signaling pathways that overlap between humans and mice.

Methods

Parotid gland tissues were harvested from 24 pSS and 16 non-pSS sicca patients and 25 controls. For mouse studies, salivary glands were harvested from C57BL/6. NOD-Aec1Aec2 mice at various times during development of pSS-like disease. RNA was analyzed with Affymetrix HG U133+2.0 arrays for human and MOE430+2.0 arrays for mouse samples. The images were processed with Affymetrix dChip software. Weighted-Gene Co-Expression Network Analysis was used to identify disease related and functional pathways.

Results

Nineteen co-expression modules were identified in human parotid tissue, 4 of which were significantly up-regulated and 3 downregulated in pSS patients compared to non-pSS sicca patients and controls. Notably, one of the human disease-related modules was highly preserved in the mouse model, and was enriched with genes involved in immune and inflammatory responses. Further comparison between these two species led to the identification of genes associated with leukocyte recruitment and germinal center formation.

Conclusion

Our systems biology analysis of genome-wide expression data from salivary gland tissue of pSS patients and from a pSS mouse model identified common dysregulated biological pathways and molecular targets underlying critical molecular alterations in pSS pathogenesis.

Introduction

Sjögren's syndrome (SS) is a chronic, inflammatory autoimmune disease characterized by lymphocytic infiltration of the exocrine glands especially salivary and lacrimal glands, leading to destruction of their functional components. The disease may exist alone as primary SS (pSS) or in conjunction with another autoimmune disorder as secondary SS (sSS) [1] . The disease affects 0.5-1.0% of the general population and shows a striking 9 : 1 female predominance [2, 3]. Although dry mouth and dry eyes are the 'hallmark' symptoms of pSS, the disease can affect almost any organ of the body and can cause substantial morbidity [4] . As yet, symptomatic therapy predominates the overall management of affected individuals [5, 6] .

The diagnostic criteria, including the American-European Consensus Group (AECG) criteria involving either serology or histopathology in conjunction with oral and ocular dryness [7] , are often inconsistently applied, are not optimal in differentiating pSS from non-pSS sicca patients or addressing extraglandular manifestations. As a result, diagnosis of Sjögren's syndrome often lags disease onset by 6-10 years. Recent attempts to address these issues, EULAR Sjögren's Syndrome Patient Reported Index: ESSPRI), and a disease activity index to evaluate systemic complications (EULAR Sjögren's Syndrome Disease Activity Index: ESSDAI) [8, 9] require further large-scale validation.

In addition to the difficulty in disease diagnosis, the underlying pathophysiologic mechanisms of Sjögren's syndrome remain obscure. A common model suggests interaction between genetic susceptibility and environmental factors such as viral infections for the immune cell activation and protracted inflammatory response resulting in glandular dysfunction and systemic autoimmunity [6] . In addition, recent studies in human and animal models have highlighted several components of the innate and adaptive immune systems as well as nonimmunologic factors [10-13]. However, there is a general lack of experimental and research intersections between humans and mouse models in terms of biological pathways and key molecular targets. Indeed, a wide variety of animal models of Sjögren's syndrome have been described; each capturing certain aspects of the disease [14]. The most widely used mouse model, NOD, exhibits CD4+ lymphocyte infiltration, autoantibodies, xerostomia and a female dominant phenotype. However, the mice also develop diabetes, which complicates the search for Sjögren's syndrome specific changes of gene expression. Recently, a modified NOD mouse model, C57BL/6.NOD-Aec1Aec2 has been described. These mice develop the symptoms of Sjögren's syndrome but not diabetes [15] suggesting that they will be a highly valuable model for understanding the pathogenesis pSS.

In the present study, we applied weighted gene co-expression network analysis (WGCNA) [16] to characterize biological pathways and molecular targets associated with pSS pathogenesis in both human parotid tissue and the C57BL/6.NOD-Aec1Aec2 mouse model. Our systems analysis of genome-wide expression data from human

(pSS) salivary gland tissue compared to salivary gland tissue from a mouse model of pSS identified common biological pathways and molecular targets that can pivotally contribute to critical molecular alterations in pSS pathogenesis.

Methods

A) Human studies

The study protocol was approved by the Institutional Review Board of the University of California at Los Angeles (UCLA) and the University Medical Center at Groningen (UMCG). All patients were recruited from the UMCG Department of Oral and Maxillofacial Surgery, were at least 21 years old, and provided written informed consent to participate in the study. The study included 24 patients who fulfilled the 2002 American-European Consensus Group (AECG) criteria for pSS [17], 16 non-sicca patients with subjective symptoms and objective signs of oral and ocular dryness but not fulfilling the AECG criteria for pSS and 25 control patients with no subjective or objective evidence of oral or ocular dryness. The patient characteristics are presented in Table 1.

Parotid gland tissue specimens

Under local anesthesia, an incisional biopsy of one parotid gland was performed as part of the diagnostic workup of the pSS and non-pSS sicca patients following AECG criteria, as described [7] , and according to the procedure described by Pijpe et al [18] . Tissue was harvested from the dorsal caudal lobe of the parotid gland from the control patients during surgery for oral or oropharyngeal squamous cell carcinoma.

After harvest, most of the specimen was snap-frozen and delivered to UCLA on dry ice and then stored at -80°C for gene expression profiling. A minor part was fixed in 4% formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin for histopathologic evaluation. The evaluation of slides from pSS and non-pSS sicca patients was performed independently by two oral pathologists, who determined the focus score (50 or more lymphocytes per 4mm^2 glandular tissue) and other characteristics such as lymphoepithelial lesions, germinal centers, fibrosis, atrophy, etc. The specimens from pSS patients showed characteristic features, for example, focus score ≥1 and presence of lymphoepithelial lesions, whereas those from non-pSS sicca and control subjects did not show any such characteristics. Histopathologic evaluation of parotid tissue from controls revealed no carcinoma.

Gene expression profiling

The isolation of total RNA from snap-frozen parotid gland tissues, in sample sizes ranging from 10-30 mg, was performed using the RNeasy kit supplied with the PARIS

Table 1. Characteristics (mean±SD) of pSS, non-pSS sicca and control subjects.*

Characteristics	pSS n=24	non-pSS sicca n=16	Controls n=25
Age (years)	49.0±15.8	52.8±15.4	59.0±12.0
Female : male	21 : 3	13 : 3	12 : 13
Ethnicity	24 Caucasian	16 Caucasian	25 Caucasian
UWS (ml/min)	0.10±0.14	0.12±0.18	NA
SWS (ml/min)	0.31±0.35	0.33±0.40	NA
Schirmer's test (mm)	9.2±7.6	14.8±11.7	NA
Focal score	3.5±1.2	0.5±1.0	0
SS-A (pos : neg)	23 : 1	2 : 14	0 : 25
SS-B (pos : neg)	19 : 5	0 : 16	0 : 25

*All pSS and non-pSS sicca patients were subjected to a complete AECG diagnostic work-up.
UWS: unstimulated whole saliva; SWS: stimulated whole saliva; NA: not assessed (in controls being head and neck cancer patients without parotid involvement, no functional tests were performed as all these patients were already scheduled for a neck dissection and functional tests were considered too much a burden to the patients at that time).

system (Ambion, Austin, TX), according to the manufacturer's protocol. Briefly, each frozen tissue specimen was homogenized in 300µl cold cell-disruption buffer and an equal volume of 2x denaturing solution was added to it. The total lysate was centrifuged for 5 min at 10,000xg to separate out the superficial aqueous phase containing RNA and then 1.25 volume of 100% ethanol was added. The mixture was loaded onto a spin column followed by washing twice with provided buffer. RNA was eluted at 100°C into 50µl of elution buffer. The resultant RNA was subjected to RNase-free DNase treatment followed by ethanol precipitation, dissolved into 15µl DNase/RNase-free water, and quantified with a Nanodrop spectrophotometer (Nanodrop Technology, Wilmington, DE).

The RiboAmp RNA Amplification system (Molecular Devices, Sunnyvale, CA) was used to perform one round of linear amplification of parotid gland tissue mRNAs taking 2.5µg total RNA from each specimen as the template. The synthesized cDNA was transcribed to cRNA and then biotinylated using the GeneChip Expression 3´-Amplification reagents (Affymetrix, Santa Clara, CA) for *in vitro* labeling. Then 15µg of labeled cRNA was fragmented into 50- to 200-bp fragments and assessed for quality with a 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA).

The fragmented biotin-labeled cRNA from pSS, non-pSS sicca and control subjects was hybridized overnight to the Affymetrix HG U133 plus 2.0 arrays. After washing to remove the unbound transcripts, the hybridized chips were stained prior to scanning. The acquired images were processed with Affymetrix dChip software and imported to the statistical program R for data analysis.

B) Mouse studies

Mouse model of SS-like disease

C57BL/6.NOD-Aec1Aec2 mice were bred and maintained under specific pathogen-free conditions within the mouse facility of the Department of Pathology at the University of Florida, Gainesville. The breeding and use of these animals for the present studies were approved by the University of Florida Institutional Animal Care and Use Committee. The animals were maintained on a 12-hour light-dark schedule and provided food and acidified water *ad libitum*.

Generation of salivary gland transcriptome data

Salivary glands were freshly excised from euthanized mice (n=5 per age group) at 4, 8, 12, 16, or 20 weeks of age, snap-frozen in liquid nitrogen, and stored at -80°C until all glandular samples were obtained. Each salivary gland was comprised of a submandibular, sublingual, and parotid gland minus salivary lymph nodes. Total RNA was isolated from salivary glands of each mouse using the RNeasy Mini-Kit (Qiagen, Valencia, CA), in accordance with the manufacturer's protocol. Hybridizations were carried out with each of the 25 individual RNA samples using Affymetrix GeneChip Mouse Genome 430 plus 2.0 Arrays (Affymetrix, Santa Clara, CA), in accordance with the manufacturer's instructions. Each GeneChip contained 45,000 probe sets that analyzed the expression level of over 39,000 transcripts and variants from over 34,000 well-characterized mouse genes.

C) Statistical procedures

Microarray data preprocessing

The Affymetrix U133 plus 2.0 microarray data were analyzed with functions and packages of the statistical software R 2.12.0 and Bioconductor 2.7. Expression intensity values were calculated for the 65 Human microarray CEL files (24 pSS, 16 non-pSS sicca and 25 controls) using the mas5 function of the Affy library. Potential array outliers were identified by studying their interarray correlation using the SampleNetwork R function. After removing potential outliers in a completely unbiased fashion, all 65 samples remained for downstream analysis. After quantile normalization, the ComBat function was used to correct for batch effects [19] . To summarize multiple probe sets per gene, we used the default settings of the collapseRows R function [20] . Thus, each of the 20718 genes on the array was represented by the probe with the highest mean expression value.

Analogous pre-processing steps were applied to the 25 mouse microarray samples corresponding to 5 different time points (weeks 4, 8, 12, 16 and 20, each time point had 5 mice). We related mouse genes to human data by using a table of orthologous genes.

Standard Differential Expression Analysis

To find differentially expressed genes between pairs of groupings (involving pSS, sicca, controls), we used 2 R functions in the WGCNA package: standardScreeningBinaryTrait and standardScreeningNumericTrait which report p-values, false discovery rates (q-values), fold changes and other widely used statistics for selecting differential expressed genes. Further, the correlation of each gene with an ordinal measure of diagnosis was assessed (0=controls, 1=Sicca, 2=pSS) which allows the identification of genes that positively or negatively increase with the disease progression in humans. We also correlated each mouse gene with time (week) to find genes that are increasing or decreasing as time progresses. These results (p-values, correlations, false discovery rate) were used to create Table 2.

WGCNA and preservation analysis

The statistical analysis software (WGCNA R package) and R tutorials for constructing a weighted gene co-expression network can be found in the literature [16, 21] . The WGCNA package first calculates all pair-wise Pearson's correlation coefficients across all samples. In a signed weighted network, the resulting Pearson's correlation matrix is transformed into a adjacency matrix ($a(ij) = |0.5+0.5*cor(x(i), x(j))|^{\beta}$) [22] . The default value of the power ß=12 facilitates a soft-thresholding approach that preserves the continuous nature of the co-expression relationships [16]. As a network dissimilarity measure we used 1 - the topological overlap measure as input for average linkage hierarchical clustering [23] . We used the dynamic branch cutting method to define modules as branches of the hierarchical clustering tree [24] . Unassigned background genes, outside of each of the modules, were denoted with the color grey.

To group the 20718 genes into modules, we used the blockwise Module R function in the WGCNA R package.

Connectivity and module membership measures

Module membership (MM), also known as eigengene-based connectivity (kME), is a measure of intramodular connectivity [25] . It is defined as $MM(i) = cor(x(i), ME)$, where x(i) is the expression profile of i-th gene and ME is the eigengene (first principal component) of the given module. We used the MM measure to select module genes for a gene ontology enrichment analysis.

Functional enrichment analysis

The Ingenuity Pathways Analysis (IPA, Ingenuity® Systems) software was used to determine whether sets of genes (e.g. preserved intramodular hub genes) were significantly enriched with known gene ontologies (GO). Ingenuity only reports uncorrected p-values.

Module preservation analysis

To evaluate which human modules could also be found in the mouse data, we used module preservation statistics implemented in the modulePreservation R function [17]. For each module in the reference data (e.g. human data), a permutation test leads to the Zsummary statistic in the test data (here the mouse data set). Zsummary>10 indicates strong evidence of preservation while Zsummary<2 indicates no preservation according to the permutation test.

Results

Identification of gene co-expression modules in parotid glands of pSS patients

A signed weighted gene co-expression network was constructed based on the 65 human parotid gland tissues (24 pSS, 16 non-pSS sicca and 25 controls). The WGCNA method clustered the 20718 human genes into 19 distinct gene co-expression modules. Since the module detection is unbiased and does not make use of gene ontology information, each of the modules was initially labeled with a unique color as an identifier (Figure 1). To define a representative module expression profile (referred to as the module eigengene), we summarized the (standardized) gene expression profiles of the module eigengene (= first principal component). The module eigengene can be considered a weighted average of the module gene expression profiles. To identify disease related modules, we correlate each module eigengene with disease status.

Strikingly, 7 out of 19 modules showed significant differential expression between pSS and control samples, which reflects the fact that thousands of genes are differentially expressed between the two groups. In particular, we found a highly significant positive correlation between disease and the Magenta (comprised of 576 genes) module eigengene ($r = 0.67$, $p < 2E\text{-}7$), the Brown (2502 genes) module eigengene ($r = 0.6$,

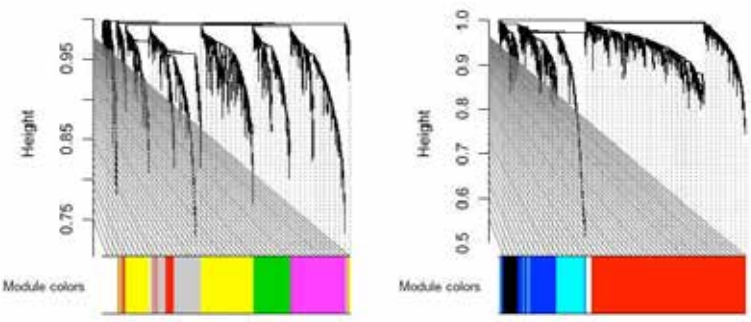

Figure 1. Detection of modules based on weighted gene-coexpression network analysis (WGCNA) of parotid gland biopsy tissue from pSS patients compared to non-pSS sicca and controls. Hierarchical cluster tree used for module detection. Modules correspond to branches of the tree and are assigned color categories as indicated by the color bands underneath the tree (see Materials and Methods).

p < 6E-6), the Light-Cyan (349 genes) module eigengene (r = 0.42, p < 0.002), and the Grey60 (based on 216 genes) module eigengene (r = 0.47, p <8E-4). Since a positive correlation indicates that the corresponding module genes are over-expressed in the diseased individuals, the Magenta, Brown, Light-Cyan and Grey60 modules are comprised of genes over-expressed in pSS samples compared to both non-pSS sicca and controls (Figure 2).

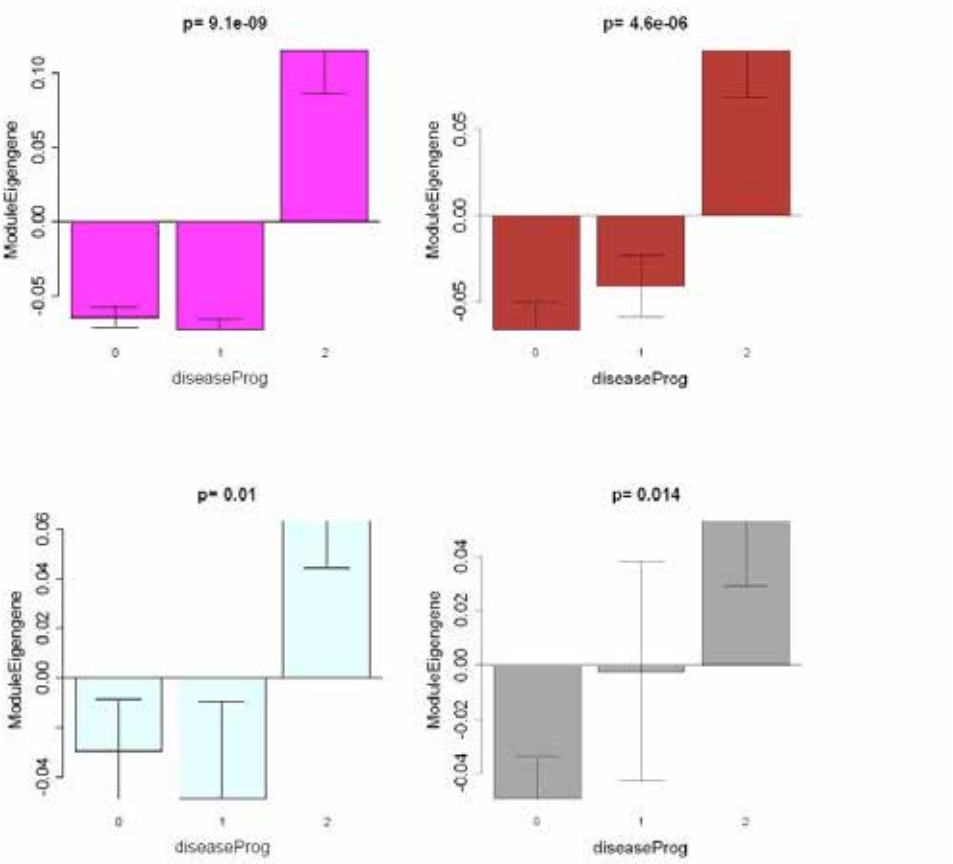

Figure 2. Identification of pSS disease-related gene modules revealed by WGCNA. The barplots show the mean value of the module eigengene (Y-axis) versus human disease progression status (X-axis) where 0 denotes healthy controls, 1 denotes non-pSS sicca patients, and 2 denotes pSS patients. The Kruskal Wallis test p-values above the plots show that the module eigengenes of all four modules, Magenta (contained 576 genes), Brown (2502 genes), Grey60 (216 genes), and Light-Cyan (349 genes), are significantly over-expressed in pSS patients compared to both non-pSS sicca patients and controls.

On the other hand, we found a highly significant negative correlation between disease status and the Turquoise (based on 3981 genes) module eigengene (r = -0.52, p < E-4), the Grey (based on 3011 genes) module eigengene (r = -0.41, p < 0.003), and the Salmon (based on 446 genes) module eigengene (r = -0.28, p <0.005). Thus, the Turquoise, Grey and Salmon modules are comprised of genes that are

under-expressed in pSS samples compared to both non-pSS sicca and controls (Figure 3). All of these p-values remain highly significant even after carrying out the most stringent multiple comparison adjustment (Bonferroni correction) for the number of modules. This co-expression module-based analysis has a major advantage over a standard differential gene expression analyses since it only relates 19 modules to pSS disease status alleviating the multiple comparison problem inherent in the raw data.

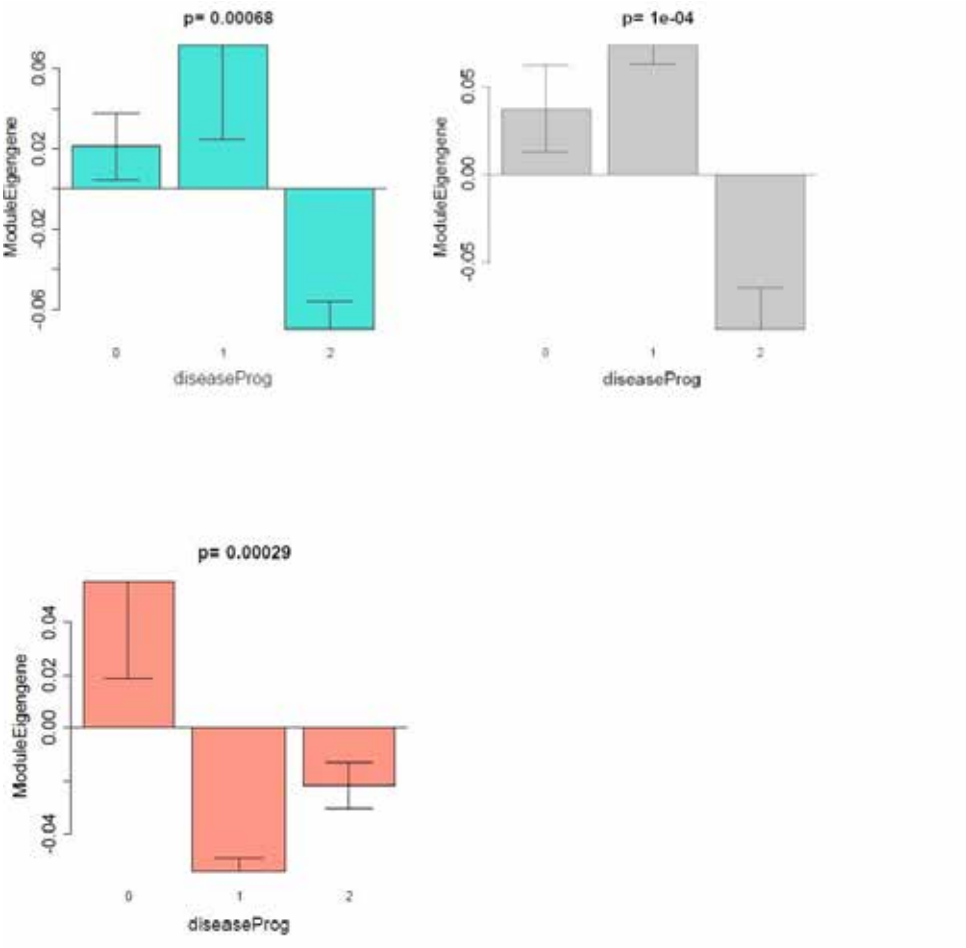

Figure 3. pSS disease-related co-expression modules that are down regulated in pSS patients. The barplots are analogous to those described in Figure 2. The module eigengenes of two modules, Turquoise (contained 3981 genes) and Grey (3011 genes), are significantly under-expressed in pSS patients compared to both non-pSS sicca patients and controls. Note that the Salmon module (446 genes) differs from the other two modules with respect to the non-pSS sicca patients, where the module eigengene is under-expressed compared to controls.

Preservation of human magenta module in a mouse SS model

We applied the modulePreservation R function to assess whether modules defined by the human data were preserved in C57BL/6.NOD-Aec1Aec2 mice, SS-like disease

model data sets. We selected the C57BL/6.NOD-Aec1Aec2 mice for the comparative gene expression analysis because it is a spontaneous disease model, probably the best-defined mouse model to date with respect to its disease profile, and has a comparative control with the same genetic background that mimics virtually all molecular and clinical aspects defined in humans. Importantly for this study, extensive transcriptomic profiling data of salivary glands defining development of SS is available. The module preservation statistics allow one to quantify which aspects of within-module topology are preserved between a reference network (human parotid glands) and a test network (mouse salivary glands). We used two composite preservation statistics (Zsummary and medianRank) to assess overall module preservation. We evaluated the preservation of the human modules in the entire mouse data set (i.e. all weeks together). Strikingly, both statistics revealed that the human Magenta module is the most highly preserved module in the mouse data (e.g. Zsummary = 11, $p < E-18$).

Furthermore, the Magenta module eigengene in the mouse data shows increasing expression across times, i.e. the Magenta genes are significantly (p=.034) over-expressed in weeks 16 and 20. These results indicates that the mouse model is appropriate when it comes to an aspect of the human disease embodied in the magenta module.

GO and pathway enrichment analysis

To study the ontology of genes in the 7 human pSS related co-expression modules, we used IPA (Ingenuity Pathway Analysis®) software. Here, we highlight results for the two most significant modules, Magenta and Turquoise.

For the Magenta module, the top-3 overrepresented subcategories within 'Gene Ontology – Biological Process' were immune response (115 genes out of 423 Magenta module genes in both human and mouse dataset, p = 1.58E-52, Bonferroni corrected p = 3.39E-49), defense response (p = 2.69E-27, Bonferroni corrected p = 5.76E-24) and inflammatory response (46 genes in both human and mouse dataset, p = 1.96E-17, Bonferroni corrected p = 4.20E-14). Additional enriched categories for the Magenta module include antigen processing and presentation (p=2.95E-17), positive regulation of response to stimulus (p=4.30E-17), Cell adhesion molecules (CAMs) (p=1.34E-13), humoral immune response (p=2.38E-13), response to wounding (p=5.68E-13), lymphocyte activation (p=2.93E-11), and cell activation (p=9.03E-11).

Strikingly, 4 of the Magenta genes are implicated the IL-4 signaling pathway (p = 0.025). Figure 4 shows an Ingenuity Pathway Analysis network plot where genes colored in grey are part of the Magenta module. Genes identified include those that encode for TLRs and their signal transduction molecule MyD88, molecules that present antigen to NK cells (e.g., members of CD1), molecules involved in immune cell recruitment and adherence (e.g., CCL21, CXCL10, CXCL12, CCR7 and SLAM7), and molecules responsible for formation of germinal centers in secondary lymphocyte organs (e.g., LTA). CXCL10 (or IP10), CXCL12 (or stromal cell-derived factor-1) and SLAM7 are

of particularly interest as they indicate recruitment of specific leukocyte subsets, in particular, macrophages, dendritic cells, T and B lymphocytes, and NK cells. We have independently validated these six genes using qPCR.

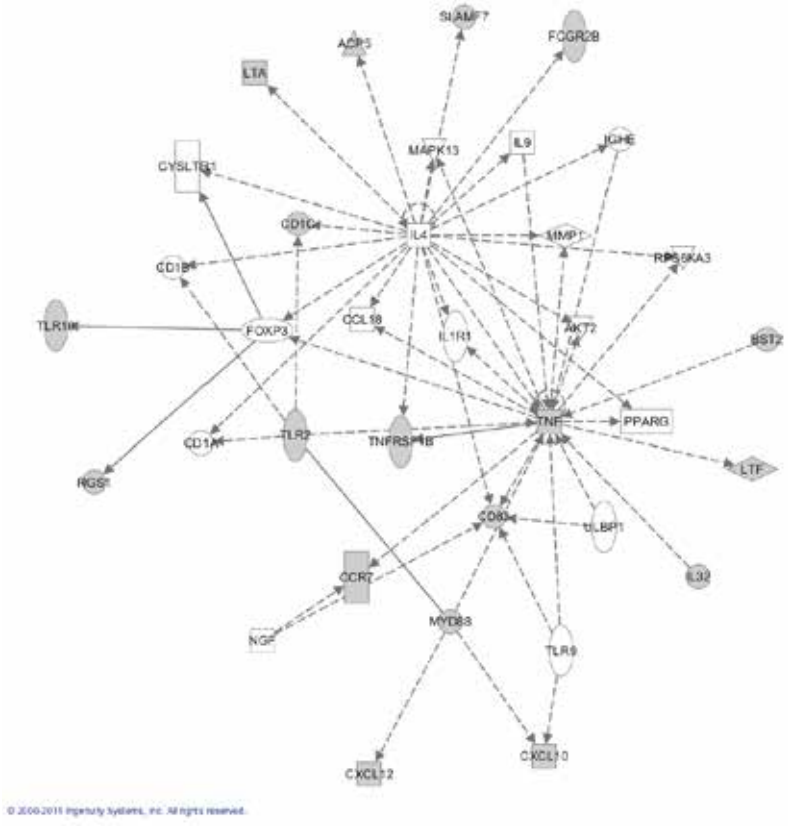

Figure 4. Ingenuity Pathway Analysis® network plot. Genes shaded in grey were part of the magenta module and are significantly altered in the parotid gland of pSS patients.

For the Turquoise module where the genes were under-expressed in pSS, the top-3 overrepresented subcategories within 'Gene Ontology – Biological Process' were Oxidation reduction (p = 7.89E-11, Bonferroni corrected p = 3.74E-7), Generation of precursor metabolites and energy (p = 4.72E-09, Bonferroni corrected p = 2.24E-5) and Cofactor metabolic process (p = 1.45E-6, Bonferroni corrected p = 0.0069). The category 'KEGG pathway' yielded the following top-3 categories: oxidative phosphorylation (p = 9.12E-7, Bonferroni corrected p = 1.77E-4), Alzheimer's disease (p = 5.66E-6, Bonferroni corrected p = 0.0011) and Huntington's disease (p = 3.69E-5, Bonferroni corrected p = 0.0071).

Table 2 reports genes with concordant progression patterns in human and mouse disease. Positively related genes were selected by requiring i) a correlation>0.5 with human progression status (defined as 0 for controls, 1 for sicca, and 2 for pSS), ii) a correlation >0.5 with mouse progression status (weeks). Similarly, we selected the negatively correlated genes using a correlation threshold of minus 0.5. Corresponding p-values are reported in the Table 2. The module membership column (MM.magenta) shows that most of these genes have concordant pattern with the magenta module eigengene illustrating again that this module contains genes that are involved in both human and mouse disease pathology.

Table 2. Genes with concordant progression patterns in human and mouse disease.

Progression	GeneSymbol	corHuman	p.Human	corMouse	p.Mouse	MM.magenta
+	ADA	0.57	5.5e-07	0.62	9.2e-04	0.70
+	AIF1	0.51	1.6e-05	0.57	3.1e-03	0.67
+	C1QB	0.54	3.2e-06	0.65	4.6e-04	0.71
+	CASP3	0.59	1.8e-07	0.52	8.2e-03	0.59
+	CYBB	0.63	1.6e-08	0.56	3.4e-03	0.82
+	ENTPD1	0.51	1.2e-05	0.62	8.5e-04	0.66
+	GZMA	0.63	1.7e-08	0.70	1.0e-04	0.72
+	GZMK	0.61	5.8e-08	0.55	4.1e-03	0.63
+	HLA-DQB1	0.53	6.0e-06	0.65	5.0e-04	0.48
+	HLA-DRB1	0.53	5.9e-06	0.74	2.6e-05	0.89
+	IFIT1	0.59	2.6e-07	0.59	1.9e-03	0.43
+	ITGAX	0.51	1.2e-05	0.63	6.8e-04	0.68
+	LY86	0.58	4.4e-07	0.53	6.4e-03	0.78
+	PLEKHA2	0.63	1.5e-08	0.52	7.1e-03	0.87
+	STAT1	0.67	7.2e-10	0.64	5.2e-04	0.60
+	TLR7	0.63	1.6e-08	0.63	6.6e-04	0.61
-	ALDH3A2	-0.50	1.8e-05	-0.63	8.0e-04	-0.64
-	ANGPTL4	-0.50	1.9e-05	-0.58	2.6e-03	-0.40
-	ATP1B1	-0.56	1.0e-06	-0.58	2.5e-03	-0.62
-	CPT1A	-0.54	4.0e-06	-0.76	8.7e-06	-0.55
-	FAF1	-0.54	4.1e-06	-0.55	4.5e-03	-0.58
-	NEO1	-0.52	9.6e-06	-0.53	6.9e-03	-0.47
-	PALMD	-0.50	2.2e-05	-0.64	6.1e-04	-0.65
-	PDHA1	-0.58	3.9e-07	-0.63	8.0e-04	-0.71
-	PPFIBP2	-0.56	1.2e-06	-0.57	3.2e-03	-0.48

corHuman denotes the correlation of the gene with human progression status(defined as 0 for controls, 1 for sicca, and 2 for pSS). corMouse denotes the correlation of the gene with mouse progression status (week). p.Human and p.Mouse report the correlation test p-values in human and mouse data, respectively. MM.magenta reports the module membership value of the gene.

Discussion

We have utilized a systems approach to identify the molecular and cellular events underlying the pathogenic process in parotid glands of pSS patients, and compared the altered expressed genes and key signaling pathways with those in the salivary glands of mice that mimic the features of pSS. With a robust statistical and bioinformatics tool, WGCNA, coupled with GO and IPA, we have clustered hundreds of co-expressed genes into 19 gene modules. Since the module definition does not make use of GO information, the modules are initially named by a color.

Our analyses highlighted 7 co-expressed modules that were enriched with genes that could discriminate pSS patients from unaffected individuals using parotid gland tissue, a prime target in SS pathogenesis. Interestingly, 4 of the 7 modules, namely Magenta, Brown, Light-Cyan and Grey60, were positively correlated with the disease status, suggesting that the genes contained in these modules are overexpressed in pSS. On the other hand, Turquoise, Gray and Salmon modules were negatively correlated with the disease status, suggesting that the genes in these modules are under-expressed in pSS.

We acknowledge that clinical samples are not gender-matched between the Sjögren's and non-Sjögren's subjects which can be a potential source of bias. We have found that gender has a negligible effect on gene expression levels in the controls. None of the genes were gender related at an FDR threshold of .05. Of the 5461 genes that were differentially expressed between pSS cases and controls at a false discovery threshold of .05, only 140 genes showed differential expression ($p<.05$) between males and females of the control samples. Further, we find a) that the vast majority of module genes are on autosomes and b) there is no evidence that gender affects their expression level in the control group.

When comparing human and mouse data, we found that the Magenta module was the most highly conserved module between these two species. Not surprisingly, this module was enriched with genes involved in immunity and inflammation, the two cardinal events in pSS pathogenesis. To our knowledge, we are the first to map out important intersects between human and mouse pertaining to key molecules and associated pathways in pSS. The knowledge gained from this work will enhance future target-based therapies for this devastating disorder. Our novel co-expression module based comparison of human and mouse models can be used to judge whether a given mouse model mirrors the human disease at the transcriptional level. Future studies could explore whether other mouse models allow for the preservation of additional human disease related modules.

Characterizing the molecular and cellular events during the progression of pSS from a healthy or even a non-pSS state remains an important challenge. Because of the lack of specific molecular markers, it is difficult to determine which healthy or sicca patientwill

progress to pSS. The gain in knowledge of these events can suggest which non-pSS individuals are at risk for developing pSS. In addition, the knowledge base could be used to intervene in the progression of disease by target-based therapies. Surprisingly, such specific regimens are not in place at present, still relying on a few trials with B cell-targeted and TNF-directed therapies evolved from testing on other autoimmune diseases [6, 10, 26-29]. Our systems-level analyses of high throughput gene expression data can demystify the critical molecular alterations in pSS pathogenesis.

WGCNA is a tool of systems biology analysis and has proven to be instrumental in identifying biological pathways and key gene constituents in a number of diseases [16, 21, 25, 30, 31]. Our module-based analysis not only alleviated the multiple testing problems inherent in microarray data analysis but also identifies biologically plausible, pSS-related modules that are highly significantly enriched with relevant GO categories.

The GO analyses have revealed striking correlations, both positive and negative, between gene modules and disease status. The most positively correlated Magenta module was significantly enriched with genes involved in antigen processing and presentation, and immune and inflammatory responses. Antigen processing and presentation are two important events in innate immunity in relation to pSS and our findings support these two phenomena [28] . Interestingly, a few Magenta genes were found to be part of the IL-4 signaling pathway, which interconnected with the TNF pathway that has been implicated in pSS disease progression. The negatively correlated Turquoise module is enriched with genes under-expressed in pSS, including those involved in oxidation reduction, generation of precursor metabolites and energy, and cofactor metabolic process. pSS patients are known to have reduced energy levels, while chronic fatigue syndrome is a common extraglandular manifestation [32] . Our data seem in line with this clinical presentation of the disease.

A unique feature of the present study is the direct comparison of the human gene expression data with that of the SS-susceptible C57BL/6.NOD-*Aec1Aec2* mouse model. Since only the human disease related Magenta module was highly significantly preserved in the mouse data, we focused on the Magenta module to identify disease relevant pathways and target genes common to both species. A majority of the identified genes were part of the immune response, whereas a minor fraction was part of the inflammatory response. Further comparison between overexpressed genes in human and increasingly expressed genes during the disease time course in mouse led to the identification of CD1, CCR7, CXCL10 (or IP10) CXCL12 (or stromal cell-derived factor-1), SLAM7 and LTA. They are of particularly interest as they indicate recruitment of specific leukocyte subsets, in particular, macrophages, dendritic cells, T and B lymphocytes, and NK cells, or involved in germinal center formation [33] . These observations provide a compelling concept that our systems approach can identify targets and pathways overlapped in human and mouse, supporting the concept that these overlapping genes and their associated pathways are critical for pSS disease

development and manifestations of clinical disease. We have independently validated these six genes using qPCR.

In conclusion, our systems approach has generated intriguing data towards identification of conserved biologic pathways and key molecules related to pSS pathogenesis, especially in parotid glands that have the potential of being therapeutic targets in this debilitating disease. The novel findings pertaining to a human-mouse intersection network will help elucidate critical pathways and molecular alterations dysregulated in pSS pathogenesis and the ability to utilize appropriate rodent models that best mimic human pathogenesis. This intersection should also permit identification of those genes and pathways that ought to be the major focus of studies in mouse models. In addition, the current results clearly point to the fact that our investigations show proof of concept that by comparing and contrasting human and rodent salivary gland WGCNA data it is possible to generate resources for future experimental as well as computational studies in evaluating the progression of pSS as revealed by human tissue analysis and then be experimentally interrogated by the most suitable mouse models as revealed by comparative WGCNA analysis.

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CHAPTER 2

CHAPTER 3

Restoration of parotid gland tissue after rituximab treatment in patients with primary sjögren's syndrome

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Abstract

Objective

To assess the effect of rituximab (anti-CD20; RTX) therapy on parotid gland immunopathology in patients who have primary Sjögren's syndrome (pSS).

Methods

In a double-blinded, placebo-controlled trial sequential parotid biopsies were taken in 20 RTX and 10 placebo treated pSS patients before and 12 weeks after RTX or placebo treatment. The relative amount (stained for CD45) and B/T-cell ratio (stained for CD3, CD20, CD79a) of the lymphocytic infiltrate as well as the relative quantity of germinal centers and lymphoepithelial lesions were assessed. At the same time interval the histological findings were correlated to secretion of stimulated parotid saliva.

Results

In all RTX treated patients a major reduction of the lymphocytic infiltrate and of the B/T-cell ratio was observed, while no reduction in infiltrate and B/T-cell ratio was observed in placebo-treated patients. The relative number of lymphoepithelial lesions and germinal centers was significantly reduced in RTX-treated, but not in placebo-treated patients. In RTX-treated patients there was no significant change in stimulated parotid salivary flow compared to baseline, while parotid flow had significantly decreased in the placebo treated patients.

Conclusion

RTX treatment leads to major reduction loss of lymphocytic infiltration, germinal centers, lympho epithelial lesions and relative B cell component. Together with this reduction, a redifferentiation of lymphoepithelial lesions into regular striated ducts was observed, reflecting major decrease of histologically verified disease activity. This reduction of inflammation in the parotid gland is reflected by a preservation of the parotid salivary flow.

Introduction

Primary Sjögren's syndrome (pSS) is a systemic autoimmune disease characterized by lymphocytic infiltration and destruction of exocrine glands, in particular lacrimal and salivary glands. The main clinical features of pSS are progressive dryness of the eyes (keratoconjunctivitis sicca) and mouth (xerostomia), concomitant with presence of a variety of systemic complications [1].

In the salivary glands the infiltrates are primarily located around striated ducts. These infiltrates are largely composed of T- and B-lymphocytes, macrophages and (plasmacytoid) dendritic cells [2, 3]. They can be organized as ectopic lymphoid tissue with segregated T- and B-cell areas and development of germinal center (GC) like structures [4]. Another histopathological characteristic is the development of lymphoepithelial lesions (LEL), formerly called "epimyoepithelial islands", triggered by intraepithelial lymphocytes [5]. LEL develop from basal cell hyperplasia of striated ducts with aberrant differentiation into a multi-layered and reticulated epithelium, devoid of luminal oxyphilic cells. This represents severe impairment of the physiological function of the ductal epithelium, especially of sodium reabsorption [5]. LELs develop intensely in the parotid gland and are sparse or absent in minor salivary glands [6]. Eventually, as the disease progresses, the acini are involved as well, leading to progressive lipomatous parenchymal atrophy [7, 8].

Immunohistologic studies of the lymphocytic infiltrate in the labial salivary gland (LSG) have revealed a predominance of T lymphocytes over B lymphocytes [9, 10]. However, recent data indicate that this might be the case for mild lesions, whereas in severe lesions B cells predominate [11].

Insights into the functions of B cells have indicated that B lymphocytes play a central role in the pathogenesis of pSS [12]. B cells become activated and undergo, presumably in the GC-like structures, antigen-driven changes such as Ig heavy chain class switching and affinity maturation. These antigen-driven changes are thought to ultimately lead to the local generation of memory B and antibody-producing plasma cells [13].
Increased activation of B lymphocytes in pSS is apparent in increased levels of serum immunoglobulins, including rheumatoid factor and autoantibodies directed to SSA and SSB antigens, accompanied by disturbances in the relative proportion of B cell subsets and the aforementioned formation of ectopic lymphoid tissue with GC-like structures in glandular tissue [3, 14, 15]. Furthermore, 5 to 10% of patients with pSS may eventually progress to develop malignant B cell lymphoma in the salivary glands, in particular the parotid glands [16, 17]. The role of B cells in the pathogenesis of pSS is further strengthened by the observed beneficial objective and subjective clinical effects of blood B cell depletion by rituximab (RTX), a chimeric monoclonal antibody that binds to the B cell surface antigen CD20 [18-21].

The LSG biopsy is used frequently as a diagnostic tool for the diagnosis of pSS [22]. However, for scientific purpose, i.e. drug monitoring, parotid biopsies are superior to LSG [22]. Furthermore an incisional biopsy of the parotid gland may cause less long term morbidity compared to LSG biopsy [6]. A further important advantage of parotid biopsy relies on the fact that the same parotid gland can be repeatedly biopsied in time and is, therefore, not only a valuable diagnostic asset but also fundamental in monitoring disease treatment at a glandular level in the same salivary gland as assessed before [6, 23].

In an open-label phase II study it was previously shown, in sequential parotid biopsies of 5 pSS patients, that RTX treatment might lead to regeneration of secretory tissue at a glandular level in responding patients [24]. This study showed a reduced lymphocytic infiltration with partial or complete loss of GC-like structures and major redifferentiation of LEL to regular striated ducts. These histopathological changes were associated with increased parotid saliva flow in patients responding to RTX treatment at a glandular level [24]. However, this study was limited due to the small number of patients and lack of a placebo group. The present study reports on the effect of RTX treatment in pSS patients based on sequential parotid biopsies obtained in a placebo–controlled, randomized clinical trial [20].

Materials and methods

Patients

Thirty patients with pSS were treated in a randomized double-blinded placebo-controlled trial on days 1 and 15 with either 1,000 mg RTX i.v. (Roche, Woerden, the Netherlands) (n= 20) or placebo i.v. (n= 10) at the University Medical Center Groningen, the Netherlands, as described before [20]. All patients fulfilled the European-US criteria for pSS [25]. To minimise side effects (infusion reactions, serum sickness), all patients, including the placebo treated patients, were pre-medicated with methylprednisolone (100 mg/i.v.), acetaminophen (1000 mg/p.o.) and clemastine (2 mg/ i.v.), and received 60 mg oral prednisone on days 1 and 2, 30 mg on days 3 and 4, and 15 mg on day 5 after each infusion. A detailed account of the patients included in this trial has been described before [20].

Serum samples were collected from all pSS patients at baseline, and at 5, 12, 36 and 48 weeks after end of RTX or placebo treatment. An incisional biopsy was taken under local anesthesia from the same parotid gland before and 12 weeks after therapy [6]. A time period of 12 weeks was chosen because at that time a total depletion of B cells was observed in serum [21]. All patients provided informed consent in accordance with the ethics committee of the University Medical Center Groningen (METC approval: 05.229).

Histopathologic analysis

Parotid gland tissue biopsies were fixed in 4% neutral buffered formalin, embedded in paraffin, cut at a thickness of 3 µm, and stained with hematoxylin and eosin. The focus score (defined as the number of mononuclear cell infiltrates containing at least 50 inflammatory cells per 4 mm^2 glandular section) and number of GC-like structures per mm^2 parotid gland tissue section was assessed. In addition, all striated ducts were evaluated, and of each duct the presence of lymphoepithelial lesions was assessed [24]. Biopsies were independently scored by 2 investigators (R.P. and S.I.) in a blinded setting. In case of discrepancy a definite scoring was established in a consensus meeting.

Immunohistochemical analysis

An anti-CD45 staining, that identifies all leukocytes, was utilized for assessment of the extent of lymphocytic infiltrate in the parotid gland.

Immunostaining was utilized for the analysis of lymphocytic infiltrate and was performed as follows. Parotid glands were fixed in formaldehyde (4%), embedded in paraffin and sectioned. Sections were stained after deparaffinisation, pre-treatment with Ultra CC1 (Ventana Medical Systems, Inc, USA), antigen retrieval and endogenous peroxidase blocking using the Benchmark machine. Sections were immunohistochemically stained with anti-CD45 (dilution 1:25, Dako, Heverlee, Belgium, clone 2B11+PD7/26), anti-CD79a (dilution 1:100, Dako, Heverlee, Belgium, clone JCB117), anti-CD20 (dilution 1:200, Dako, Heverlee, Belgium, clone L-26) and anti-CD3 (dilution 1:20, Monosan, Uden, the Netherlands, clone PS-1) antibodies. The sections were then treated with peroxidase-labelled secondary antibody and visualized with the chromogen DAB (3,3' Diaminobenzidine) solution. The relative amount of CD45-positive lymphocytic infiltrates was assessed in relation to the total amount of tissue parenchyma by morphometry with use of ImageJ software (v1.46). Furthermore, in representative areas of lymphocytic infiltrates the number of B cells (CD20) and T cells (CD3) was estimated among 1000 lymphocytes per infiltrate and consecutively calculated as B/T-cell ratio. To evaluate a possible down-regulation of CD20 antigen on persisting B cells due to anti-CD20 therapy, quantification of B cells was performed not only with CD79a but also with antibodies to CD20.

A double immunohistochemical labeling technique for cytokeratin 14 (CK14;Serotec, Düsseldorf, Germany; expressed by basal cells of striated ducts and myoepithelial cells in the acini) and Ki67 (Dianova, Hamburg, Germany) was performed as described previously [26] in order to improve exact localization of cellular proliferation in different types of epithelial cells.

Parotid gland function

Stimulated parotid saliva was collected in a standardized way at baseline and at 12 weeks after treatment as described previously [21] .

Statistical analysis

The Wilcoxon matched pairs test was used for intra-individual comparison of pre- and post-treatment values. Data are presented as the mean or median. A value of $P<0.05$ was considered statistically significant.

Results

From the 30 patients, six patients had to be excluded from histopathological analysis, either due to serum sickness (n=1, RTX-group) or the full use of the parotid biopsy sample at baseline for diagnostic purposes (n=4, RTX-group; n=1, placebo group). Thus, evaluation could be performed of parotid glands taken from 15 RTX treated patients and 9 placebo treated patients.

Lymphocytic infiltrate in parotid glands

While focus scores remained at the same level in both treatment groups (figure 1a), the far more accurate measurement of the amount of lymphocytic infiltrate, using anti CD45 staining, clearly demonstrates a significant decrease (P= 0.015, figure 1b) at 12 weeks after RTX treatment. In the placebo group no change in lymphocytic infiltrate was observed between baseline and 12 week post-treatment values (P= 0.426, figure 1b).

The amount of B cells based on staining for CD79a and CD20 did not differ between both groups. A subsequent analysis of the B/T-cell ratio showed that at baseline 58% of the cells in the infiltrates in the parotid glands of pSS patients were B cells and 42% T cells. Twelve weeks after RTX treatment the B/T-cell ratio was reversed (36% B cells versus 64% T cells), thus showing a significant decrease in the relative percentage of B cells in the infiltrates (P=0.0089, figure 2). By calculating the amount of B and T cells from the lymphocytic infiltrate, it was observed that there was an absolute decrease in the amount of B cells (from 11.1×10^5 μm^2 to 3.4×10^5 μm^2) as well as in the amount of T cells (from 8.2×10^5 μm^2 to 5.9×10^5 μm^2) at 12 weeks after rituximab treatment compared to baseline.

Analysis of the B/T-cell ratio in the placebo group showed that 66% of B cells and 33% of T cells were present in the infiltrates of parotid glands at baseline. Percentages of B and T cells remained unaffected at 12 weeks after treatment (62% versus 38%, respectively). This notion was confirmed by calculation of the total amount of B cells (from 27.2×10^5 μm^2 to 31.2×10^5 μm^2) and T cells (from 13.6×10^5 μm^2 to 15.3×10^5 μm^2)) at baseline compared to 12 weeks after placebo treatment .

Figure 1A: Focus score

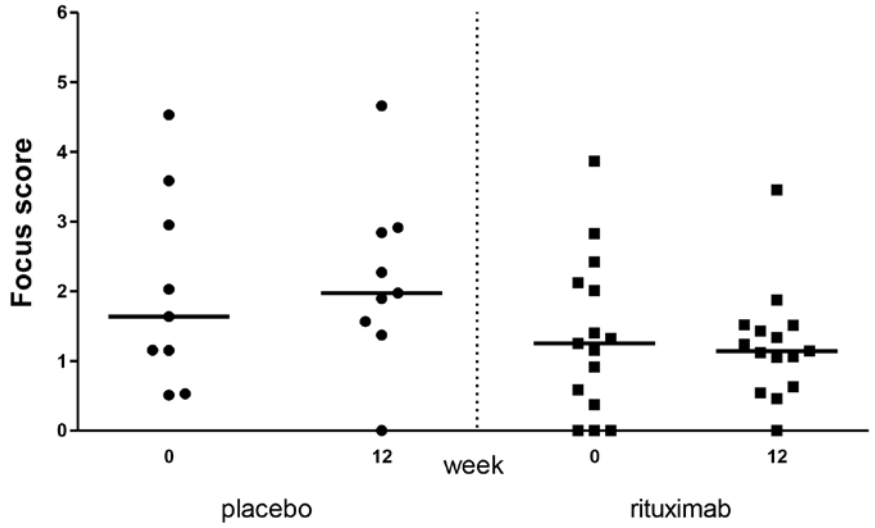


Figure 1B: Relative area of infiltrates

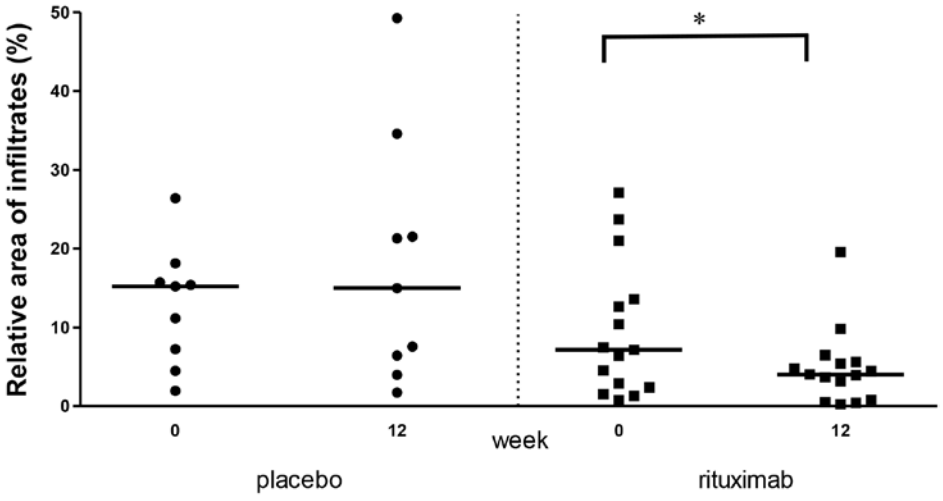


Figure 1. Effect of placebo (n=9) and RTX (n=15) treatment on lymphocytic infiltrate in parotid glands of pSS patients. *indicates significant difference compared with baseline values (P<0.05). Horizontal lines indicate median values.
A. Focus score in parotid gland tissue.
B. Relative area of infiltrates in parotid gland tissue assessed with CD45 staining.

Figure 2A: Parotid biopsy specimen before RTX treatment.

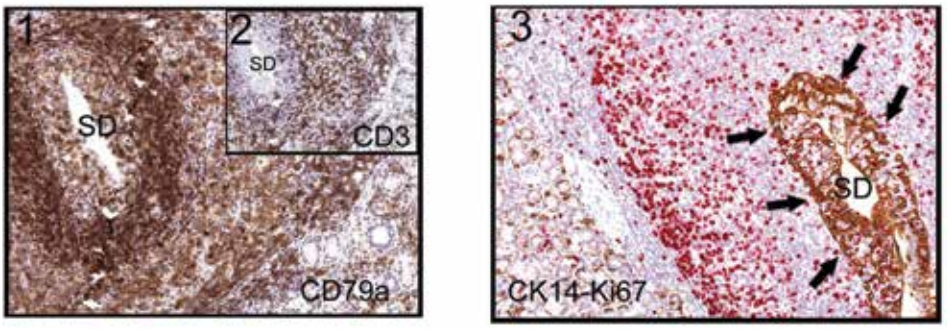
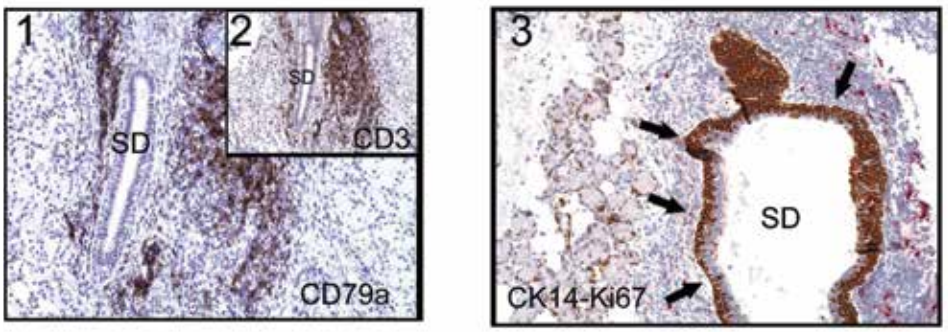


Figure 2B: Parotid biopsy specimen 12 weeks after RTX treatment.



A. Immunohistochemistry for CD79a B cells (panel 1, brown), CD3 T-cells (panel 2, brown) and CK14-Ki67 localization (panel 3, brown) before RTX treatment in parotid gland tissue. Dense lymphocytic infiltration was mostly located around the striated duct (SD) and consisted of CD3 cells and CD79a positive B cells. In panel 3, a striated duct is transformed into fully-developed lymphoepithelial lesion (LEL) with numerous intraepithelial lymphocytes (arrows).

B. Immunohistochemistry for CD79a B cells (panel 1, brown), CD3 T cells (panel 2, brown) and CK14-ki67 localization (panel 3, brown) 12 weeks after RTX in parotid gland tissue. When compared to the specimen showed in figure 3a, a profound reduction in periductal lymphocytic infiltration, especially of B-lymphocytes, is clearly visible. Increase of the number of regular striated ducts with CK14 positive basal cells and CK14-negative luminal oxyphilic cells (arrows).

Figure 3A: Lymphoepithelial lesions

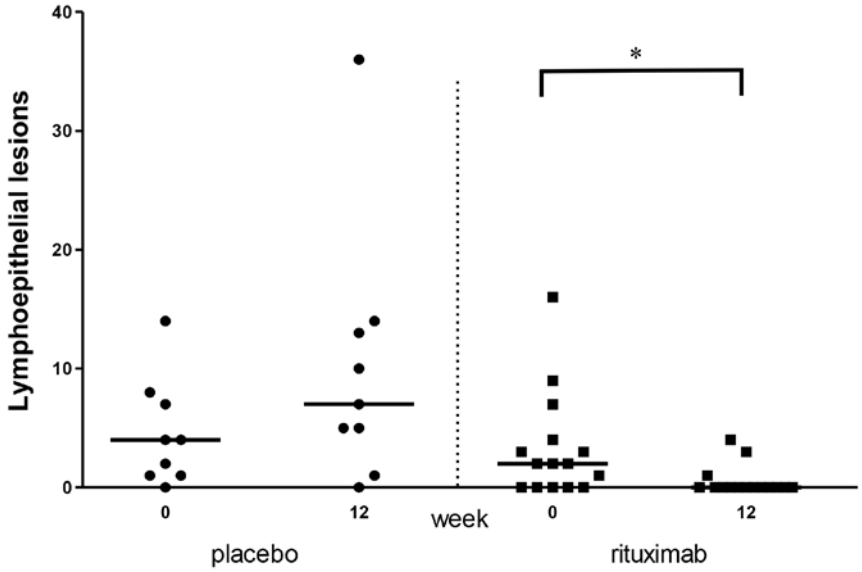


Figure 3B: GC-like structures

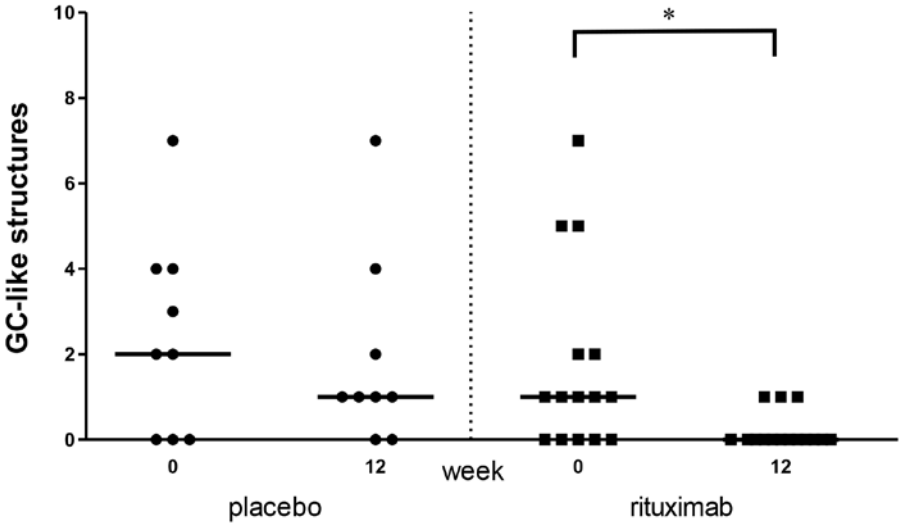


Figure 3. Amount of LELs and GC-like structures in parotid glands of placebo (n=9) or RTX treated (n=15) patients. *indicates significant (P<0.05) difference compared with baseline values. Horizontal lines indicate the median values.
A. Number of LEL in parotid biopsies.
B. Number of GC-like structures in parotid biopsies.

Lymphoepithelial lesions and germinal centers

In the RTX treated group a significant decrease in amount of LEL was observed after 12 weeks of treatment (P= 0.025; figures 2, 3a). Twelve out of 15 cases were even completely devoid of LEL and showed exclusively histomorphological regular striated ducts, after RTX treatment. In the placebo group no change in amount of LEL was observed after 12 weeks (P= 0.155; figure 3a).

GC-like structures were present in 68% of parotid glands at baseline. The RTX treatment group showed a significant decrease in total amount of GC-like structures (per surface unit) (P= 0.049; figure 3b) after RTX treatment. Twelve out of 15 cases were completely devoid of GC-like structures 12 weeks after treatment. In the placebo group no significant difference was observed in the total amount of GC-like structures (P= 0.525; figure 3b) between baseline levels, and 12 weeks after treatment.

CK14-Ki-67 double staining showed sparse staining for Ki-67 in ductal basal cells indicative of low proliferation before and after RTX treatment (figure 2b). Furthermore, proliferation of acinar cells in the parotid glands in both treatment groups was comparable before and after treatment (rituximab from 2.2% to 2.1% and placebo 2.1% to 2.2%).

Parotid gland function

In the RTX treated group no significant difference was observed in stimulated parotid salivary flow at 12 weeks in comparison to baseline (figure 4). In marked contrast, however, in placebo treated patients stimulated parotid salivary flow had significantly decreased 42% at 12 weeks after placebo (P=0.039).

Figure 4: Parotid salivary flow

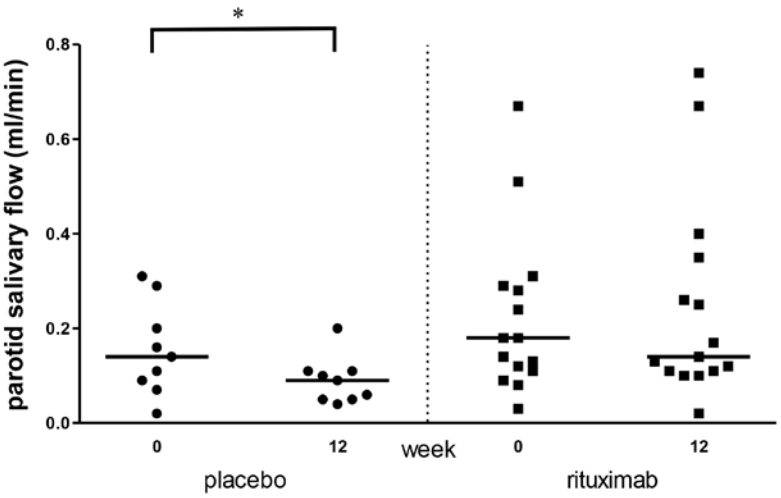


Figure 4. Effect of placebo (n=9) and RTX (n=15) treatment on parotid salivary secretion in pSS patients. *indicates significant difference compared with baseline values (P<0.05). Horizontal lines indicate median values

Discussion

The results of the current placebo controlled study showed that RTX treatment is able to significantly reduce overall lymphocytic infiltrate with major loss of B cell component and GC-like structures. In addition, a major reduction of LELs as a sign of structural regeneration of the glands was observed. Therefore, it was concluded that, targeting B cells in pSS leads to a major decrease in histologically verified local inflammation state, demonstrating the crucial role of B cells in this autoimmune disease. Although RTX treatment results in the almost complete absence of B cells in the serum of patients with pSS, this is not mirrored by a complete absence of B cells in parotid salivary gland tissue indicating that RTX does not fully depletes B cells in salivary (parotid) glands of pSS patients.

The lack of total depletion of B cells in the salivary glands could be responsible for the relapse of pSS disease activity. These results are in line with other studies in pSS [27] and RA [28-30] showing persistence of B cells in the local tissue after RTX treatment. Devauchelle-Pensec et al. reported a total depletion in B cells in LSG samples of pSS patients after RTX treatment [31]. The reason for the discrepancy between the latter results and the findings in the current study is unclear. Possible explanations may include the difference in sample size or the notion that GC-like structures are more frequently present in parotid glands compared to LSGs [32]. These GC-like structures can act as a niche with restricted access to RTX, leading to the persistence of B cells after treatment [33, 34].

The exact mechanism regarding possible regeneration of ductal epithelium from LEL is not known. LELs in pSS develop from enhanced proliferation of basal cells of striated ducts with an aberrant metaplastic lymphoepithelial differentiation, triggered by the epitheliotropic autoimmune inflammation [5]. Studies from the intestinal epithelium indicate that cytokines are key regulators of epithelial homeostasis (reviewed by Koch and Nusrat, 2012) [35]. For example, IFN-γ and TNF-α are central mediators of disruption of the epithelial barrier function of the intestine. Also, the integrity of the ductal and acinar epithelium of salivary gland tissue is affected by these cytokines by disorganization of the tight junctions [36].

It can be assumed that pro-inflammatory cytokines, such as IL-6, IFN-α and TNF-α trigger the basal cells to proliferation and differentiation. Furthermore, it was recently demonstrated that pro-inflammatory cytokines, such as IL-6, IFN-α and TNF-α, decrease after RTX treatment [37]. This decrease in pro-inflammatory cytokines could explain the regeneration of ductal epithelium from LEL.

According to the immunohistologic improvement, some clinical improvement in parotid flow after RTX treatment was expected. In contrast to the findings in 3 out of 5 patients in the previous pilot study [24], parotid salivary flow was not significantly

increased after RTX treatment in the current study but remained at the baseline level. Importantly, however, parotid flow in placebo treated subjects reduced during the same observation period. This reduction of parotid flow in the placebo treated patients is in line with the findings on the natural progression of salivary gland dysfunction in pSS patients [38]. Increased apoptotic loss and gradual dedifferentiation of acinar cells contribute to gradual parenchymal atrophy in pSS [7, 39-41]. After RTX treatment the observed low proliferative capacity of the salivary acinar parenchyma is obviously not able to compensate for the inflammation-induced parenchymal cell loss in most patients. This might explain the observed lack of improvement in parotid salivary flow in the current study, despite of profoundly reduced lymphocytic infiltrate.

In conclusion, RTX treatment leads to major reduction of lymphocytic infiltration and relative B cell component with major loss of GC-like structures and with redifferentiation of LELs into regular striated ducts, reflecting major reduction of inflammation. This reduction is reflected by a preservation of the parotid salivary flow.

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CHAPTER 3

CHAPTER 4

Serum levels of BAFF, but not APRIL, are increased after rituximab treatment in patients with primary Sjögren's syndrome

This chapter is an adapted version of:

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Ann Rheum Dis. 2013; 72: 146-8

Abstract

Objective

To assess the effects of rituximab (RTX) treatment in primary Sjögren's syndrome (pSS) patients on B cell activating factor (BAFF) and proliferation-inducing ligand (APRIL).

Methods

In a randomised double-blinded placebo-controlled trial, pSS patients were treated on days 1 and 15 with B cell depleting therapy with RTX (n=20) or placebo (n=10). Serial blood samples were collected from the pSS patients at various time intervals (0, 5, 12, 36 and 48 weeks) following RTX/placebo treatment and from 10 age- and sex-matched healthy controls (HCs). Parotid gland biopsies were taken from pSS patients at baseline and at 12 weeks after RTX/placebo treatment, to analyse presence of BAFF in glandular tissue before and after treatment. Numbers of B cells were examined in peripheral blood from pSS patients and HCs by four-color flow cytometry. Serum levels of BAFF and APRIL were measured by enzyme-linked immunosorbent assay.

Results

Complete depletion of circulating B cells was observed in patients treated with RTX. Partial reconstitution of B cells started after 12 weeks following RTX treatment. At baseline, BAFF and APRIL serum levels were significantly increased in pSS-patients compared to HCs ($P<0.05$). Following RTX treatment, BAFF levels increased up to 12 weeks after RTX treatment (3-4 fold, $P<0.05$), where after BAFF levels gradually returned to baseline values. In contrast to BAFF, no changes in serum levels of APRIL were observed after RTX treatment.

Conclusion

RTX treatment of pSS patients results in a significant increase of serum levels of BAFF, whereas APRIL levels are unaffected. These findings may have therapeutic implications.

Introduction

Primary Sjögren's syndrome (pSS) is characterized by mononuclear infiltrates, mainly composed of B- and T-lymphocytes, in exocrine glands. In particular, lacrimal and salivary glands are affected leading to destruction of these glands, which results in dryness of mouth (xerostomia) and eyes (keratoconjunctivitis sicca) [1].

Involvement of B-lymphocytes in pSS is apparent considering the presence of autoantibodies, including rheumatoid factor and antibodies directed against SS-A/Ro and SS-B/La antigens, and hypergammaglobulinemia [1, 2]. B cell involvement is further demonstrated by the presence of B cell infiltrates with germinal center-like structures in affected tissues and aberrant distribution of B cell subsets in the peripheral blood [3, 4]. B cell involvement is also illustrated by the notion that 5-10% of patients with pSS develop MALT lymphoma [5].

In line with an important role for B cells in pSS, B cell depletion using rituximab (RTX), a chimeric monoclonal antibody that targets the pan-B-lymphocyte antigen CD20, appears to be effective in the treatment of patients with early diagnosed pSS [6-8]. Clinical effects persisted for 24-36 weeks, a time period during which B cells in the peripheral blood were reduced. Reduction in glandular inflammation and redifferentiation of duct lesions to regular striated ducts together with an increase in parotid flow was demonstrated at 12 weeks after rituximab treatment [6, 9, 10]. Together these data suggest that B cells are involved in inducing lesions in glandular tissue in pSS.

B cell activating factor (BAFF) and proliferation-inducing ligand (APRIL), both members of the tumor necrosis factor (TNF)-ligand family, are important cytokines involved in B cell survival and activation [11]. These cytokines are thought to play significant roles in the pathogenesis of various autoimmune diseases including pSS [12, 13]. Binding of BAFF to the BAFF receptor (BAFF-R) induces survival signals for B cells [14]. As such, signalling of the BAFF-R positively regulates B cell homeostasis. BAFF can also bind to another receptor; the transmembrane activator and calcium-modulator and cyclophilin ligand interactor (TACI), to which also APRIL can bind. Interaction of BAFF or APRIL to TACI leads to B cell activation resulting in T cell independent isotype switching and in production of immunoglobulins [15, 16]. Finally, in particular APRIL contributes to the maintenance/ survival of long-lived plasma cells by interaction with the receptor B cell maturation antigen (BCMA) on plasma cells [17, 18]. Studies in BAFF transgenic mice demonstrated that excessive BAFF production results in autoimmunity, caused by a less stringent selection of immature/transitional B cells and survival of low/intermediate affinity self-reactive B cells [19-21]. Importantly, these BAFF-transgenic mice develop severe autoimmune symptoms that are similar to systemic lupus erythematosus (SLE) and pSS as found in human [17-20].

BAFF and APRIL levels in serum and saliva are elevated in patients with pSS compared to healthy controls [22]. The origin of these cytokines is not completely understood. A recent study conducted by Yoshimoto et al., revealed that circulating monocytes from pSS patients produced higher amounts of BAFF than normal monocytes, even in the absence of stimulation [23]. In salivary glands of pSS patients, both ductal and infiltrating cells appear to produce BAFF, which might be induced by virus, TLR signalling, or by type I Interferon (IFN) [24, 25]. Furthermore, levels of both APRIL and BAFF correlate with serum IgG levels in pSS-patients and BAFF levels are higher in patients with anti-SSA and anti-SSB antibodies [13, 26, 27], denoting their possible role in disease progression. Together these observations demonstrate that BAFF and APRIL could be important players in the pathogenesis of pSS.

BAFF levels rise after RTX treatment of patients with rheumatoid arthritis and SLE [28, 29]. Limited data are currently available on BAFF levels in pSS patients treated with RTX. Lavie et al. have demonstrated a rise in BAFF levels after RTX treatment in two pSS patients with one time point after RTX treatment [30]. Effects on APRIL levels in pSS after RTX treatment are lacking. With regard to the strong impact of BAFF and APRIL on B cell survival and activation, it is therefore of importance to study in detail the effects of RTX-induced B cell depletion on serum BAFF and APRIL levels in patients with pSS. For this reason we assessed here in a double-blind, randomised, placebo-controlled trial the effects of RTX treatment in pSS patients on serum BAFF and APRIL levels up to 48 weeks after RTX treatment. In addition we analyzed whether BAFF is still produced in the affected glandular tissue, despite the RTX treatment.

Materials and methods

Patients and controls

Thirty patients with pSS had been treated in a randomised double-blinded placebo-controlled trial on days 1 and 15 with either 1,000 mg RTX (Roche, Woerden, The Netherlands) (n= 20) or placebo (n= 10) at the University Medical Center Groningen, The Netherlands [6]. All patients fulfilled the European-US criteria for pSS [31]. To minimize side effects (infusion reactions, serum sickness), all patients were pre-medicated with methylprednisolone (100 mg/i.v.) and received 60 mg oral prednisone on days 1 and 2, 30 mg on days 3 and 4, and 15 mg on day 5 after each infusion with rituximab. A detailed account of the patients included in this trial has been described before [6]. From the 30 patients, two patients were excluded from analysis, either due to serum sickness (n=1, RTX-group) or insufficient serum sample at baseline (n=1, placebo group). In addition to the pSS patients, 10 age- and sex-matched healthy controls (HCs) were included in the current study (1 male and 9 females; mean age 46 years [range 29–60 years]). All patients and controls provided informed consent

in accordance with the ethics committee of the University Medical Center Groningen (METC approval: 05.229).

B cell analysis

Estimation of B cell numbers was performed on fresh blood samples from HCs and from pSS patients at various time points (baseline, 5, 12, 36 and 48 weeks after RTX/placebo treatment) with use of FACS-Calibur flow cytometer as previously described [32].

Quantification of soluble BAFF, APRIL and IgG

Serum samples were collected from all pSS patients at various time points (at baseline, and at 5, 12, 36 and 48 weeks after rituximab treatment) and from 10 age- and sex-matched HCs. Concentrations of BAFF and APRIL in serum were measured by enzyme-linked immunosorbent assay (ELISA) using commercial kits from R&D systems (Minneapolis, USA) and Bender Med Systems (San Diego, USA), respectively, following the manufacturer's instructions. The minimal detectable concentration of BAFF-ELISA was 3.38 pg/mL and for APRIL-ELISA was 400 pg/mL. Levels of IgG were measured by laser nephelometry.

Immunohistochemical staining of BAFF in parotid gland tissue

Serial parotid biopsies were collected before and 12 weeks after the first infusion of RTX/placebo as previously described [10, 33]. To analyse whether BAFF was still produced after RTX treatment, we examined biopsies of 10 patients (5 rituximab, 5 placebo) for BAFF expression. Cryostat sections (5 µm) were fixed with acetone and incubated with 10 µg/mL rat anti-human BAFF (Buffy 2) (Abcam, Cambridge, UK). Sections were subsequently washed with phosphate buffered saline (PBS) and incubated with biotin labeled goat anti rat Ig (1:50; BD Bioscience, San Diego, USA) supplied with 1% normal human serum. After rinsing, horseradish peroxidase labeled avidin (1:50; BD Bioscience) and 3,3′-diaminobenzidine substrate (Sigma Chemical Co., St. Louis, Missouri, USA) were used for visualization. Sections were counterstained using hematoxylin (Sigma Chemical Co., St. Louis, Missouri, USA). After dehydration with alcohol, slides were mounted in DePeX mounting medium (VWR International, Amsterdam, the Netherlands). Immunostained slides were evaluated by light microscopy.

Statistical analysis

A linear mixed effect analysis test (SPSS version 18.0) was used to analyze the difference of BAFF/APRIL serum levels between treatment groups. Analysis was performed using time and treatment as variables. The paired t-test was used for intra-individual comparison of values at different time points. Spearman Rank Correlation Coefficient test was used for detection of a significant relationship between serum BAFF and B cell

levels. A log transformation prior to statistical analysis was performed, if data were not normally distributed. Data are presented as the mean or median and range. A value of $p<0.05$ was considered statistically significant.

Results

RTX treatment results in complete depletion of B cells in peripheral blood

As previously described in detail elsewhere, peripheral blood B-lymphocytes were completely depleted in patients treated with RTX [32]. Partial reappearance of B-lymphocytes started 12 weeks after treatment. In patients treated with placebo a drop in B-lymphocyte levels was only visible at 5 weeks, most likely due to the effect of corticosteroids given to all pSS patients to prevent adverse events.

Figure 1: BAFF and APRIL levels

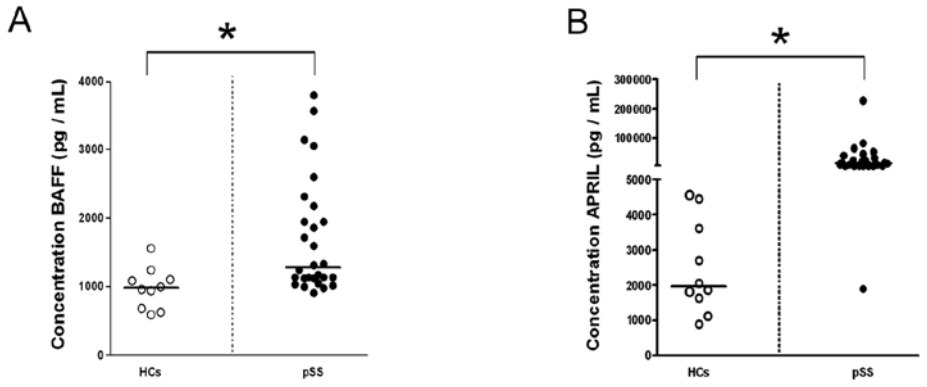


Figure 1. BAFF and APRIL levels in HCs and pSS patients at baseline. *indicates significant differences between HCs and pSS subjects ($P<0.05$). The horizontal lines indicate the median values.
A. BAFF levels
B. APRIL levels

BAFF levels increase after RTX treatment

At baseline, serum BAFF levels were significantly higher (1.3 fold) in pSS patients (median 1277 pg/mL [range 906.9-3802 pg/mL]) compared to HCs (median 982.6 pg/mL [range 599.9-1564 pg/mL]); $P<0.01$; figure 1a). These findings are in line with observations by others [22]. In RTX treated patients a 3-4 fold increase in BAFF levels ($P<0.001$) was observed up to 12 weeks after treatment followed by a gradual return towards baseline levels (figure 2a). When B cell numbers declined as a consequence of RTX treatment, BAFF levels rose significantly, and when B cells reappeared, BAFF levels decreased again towards their baseline values [6]. Indeed, correlation analysis confirmed the inverse relation between peripheral B cell numbers and BAFF levels

Figure 2: BAFF and APRIL levels in pSS patients before and after treatment

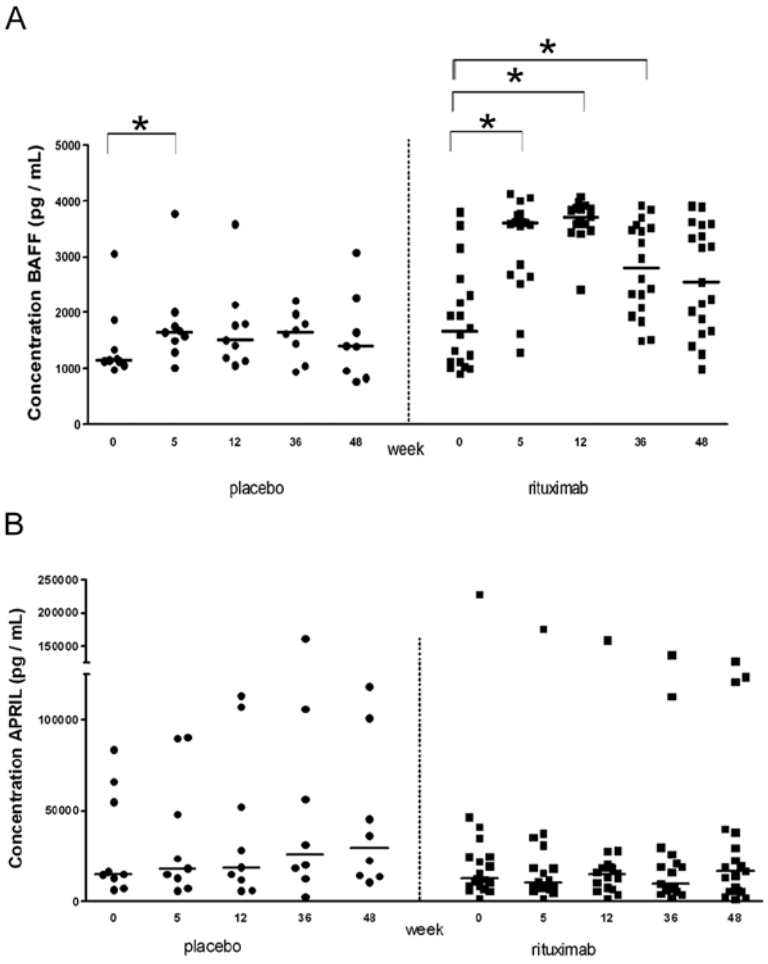


Figure 2. Effect of placebo and RTX treatment on BAFF and APRIL levels in pSS patients. *indicates significant differences compared with baseline values (P<0.05). The horizontal lines indicate the median values.
A. BAFF levels
B. APRIL levels

in serum (P<0.0001). In contrast to RTX treated patients, in placebo treated patients only a slight, but significant increase in BAFF levels, was detected at 5 weeks after treatment (P<0.001), but not at other timepoints. At this time point B cell numbers were slightly decreased. A linear mixed effect analysis confirmed a significant treatment effect between RTX and placebo treated patients (P= 0.02) during the studied time period.

APRIL levels are unaffected after RTX treatment

At baseline, not only BAFF levels, but also APRIL levels were significantly higher (7-8 fold) in serum of pSS patients (median 15098 pg/mL [range 1891-228591 pg/mL]) compared to HCs (median 1965 pg/mL [range 889-4567 pg/mL]; P<0.05; figure 1b), as has been described previously [26]. In marked contrast with BAFF, no changes over time were observed in serum APRIL levels in RTX or placebo treated subjects during follow up (figure 2b). There was a remarkable variation in APRIL levels at baseline. This was clearly a characteristic of the individual patients since patients with high baseline values of APRIL remained high during the entire time period studied. One of the pSS patients had very high APRIL levels (8-fold) at baseline (figure 2b). After RTX treatment APRIL levels in this patient remained persistently high. There was no significant difference in APRIL levels between the placebo and RTX group over the time period studied (P=0.054, linear mixed effect analysis), even not when the patient with the very high APRIL levels (in the RTX group) was omitted from the analysis.

Figure 3A: immunohistochemistry for CD79a B cells (1), CD3 T cells (2) and BAFF (Buffy-2) localization (3) in pSS patients at baseline.

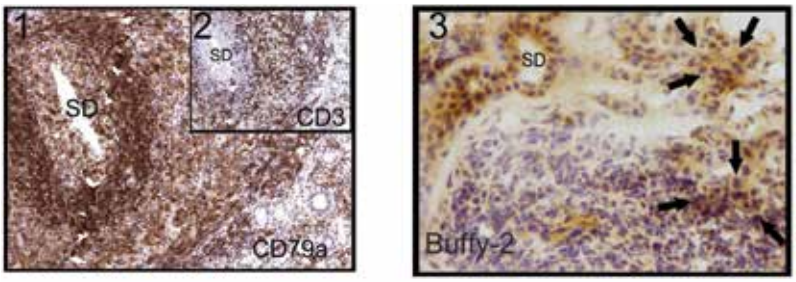
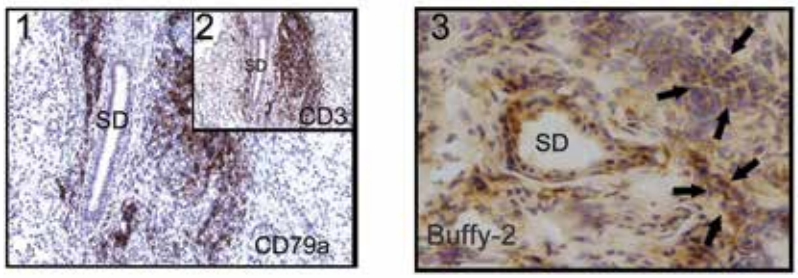


Figure 3B: immunohistochemistry for CD79a B cells (1), CD3 T -cells (2) and BAFF (Buffy-2) localization (3) in pSS patients 12 weeks after RTX treatment.



A. Immunohistochemistry for CD79a B cells (3A-1, brown), CD3 T cells (3A-2, brown) and BAFF (Buffy-2) localization (3A-3, brown) before RTX in parotid gland tissue. Lymphocytic infiltration was mostly located around the striated duct (SD) and consisted of CD3 T cells and CD79a positive B cells. BAFF is predominantly localized in the lymphocytic infiltrate (arrows).
B. Immunohistochemistry for CD79a B cells (3B-1, brown), CD3 T cells (3B-2, brown) and BAFF (Buffy-2) localization (3B-3, brown) after RTX in parotid gland tissue. A reduction in lymphocytic infiltration around the striated duct (SD) was visible after RTX. BAFF is predominantly localized in the lymphocytic infiltrate (arrows).

Figure 4: Serum IgG levels

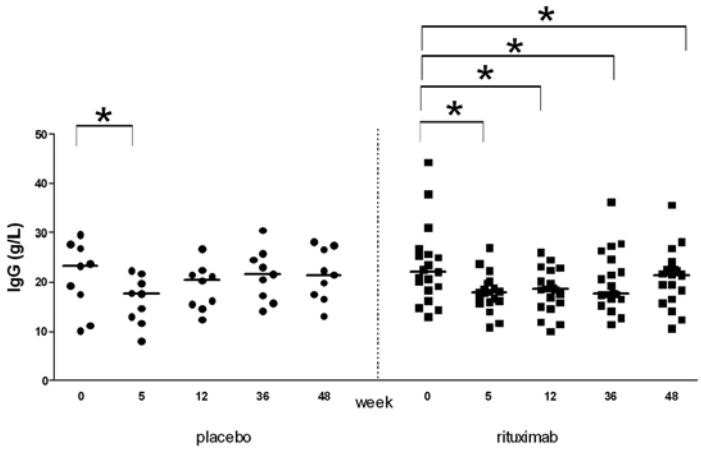


Figure 4. Serum IgG levels of placebo/ RTX treated patients. *indicates significant (P<0.05) differences compared with baseline values. The horizontal lines indicate the median values.

BAFF is expressed in parotid glands after RTX treatment

Immunohistochemistry of parotid gland samples showed the presence of lymphocytic infiltrates in all patients at baseline. The lymphocytic infiltrates were predominantly located around the striated ducts and consisted predominantly of T cells (CD3+) and B cells (CD79a+) (figure 3A1-2). At 12 weeks after treatment the lymphocytic infiltrates were clearly smaller in size in the RTX treated group, but not in the placebo treated group (figure 3B1-2). Staining for BAFF showed the presence of this cytokine, both in tissue sections obtained at baseline and those at 12 weeks after RTX or placebo treatment. In all tissue sections BAFF was located predominantly in the lymphocytic infiltrates surrounding the ducts. Despite the reduction in size of the lymphocytic infiltrates no clear changes in BAFF staining were observed after RTX treatment (figure 3A3, B3).

Decreased serum levels of IgG after RTX treatment

BAFF (and APRIL) are also involved in B cell activation and higher BAFF levels could therefore result in higher IgG serum concentrations observed in pSS patients [6, 34]. As expected, at baseline, patients with pSS had higher IgG levels compared to healthy controls. However, in contrast to Mariette et al. [34], there was no positive correlation between baseline levels of BAFF and IgG (R=-0.3547, P=0.0695). After RTX treatment, levels of IgG decreased significantly for a prolonged period of time (figure 4). Meanwhile, levels of BAFF increased significantly, compared to baseline values (figure 2a). In placebo patients IgG levels were only decreased, at 5 weeks after treatment, at a timepoint that BAFF levels were also significant increased. This was most likely due to the effect of corticosteroids given against adverse effects (figure 2a).

Discussion

Although intervention treatment options for pSS are currently very limited, B cell depletion with RTX appears to be an effective modality [6, 10]. Treatment with RTX results in an effective depletion of B cells in the peripheral blood and thus interferes with B cell homeostasis. Since BAFF and APRIL are two critical cytokines for the control of B cell homeostasis, we studied the effects of RTX treatment on the serum levels of these cytokines in pSS patients. We observed in a placebo controlled trial that the already higher baseline levels of serum BAFF in pSS patients further increase after RTX treatment, when B cells were absent. BAFF levels declined, when B cells reappeared. In marked contrast, APRIL levels, which partly target the same receptors as BAFF, were largely unaffected, and remained high after RTX treatment.

A likely explanation for the increase in BAFF levels after RTX treatment is that depletion of B cells also results in removal of BAFF-R which is highly expressed on mature B cells. Expression of BAFF-R is low on newly formed, immature, B cells and is upregulated when these cells become mature B cells [35]. Absence of mature B cells results in absence of available BAFF-R leading to an increase in free, unbound BAFF in serum. Indeed, in line with this notion, decline in serum BAFF levels coincides with the reappearance of B cells in blood after RTX treatment [32]. In addition to this mechanism, Lavie et al. [30] also observed that after RTX treatment mRNA levels encoding for BAFF in peripheral blood mononuclear cells are higher than before treatment. Apparently, in addition to an increase in BAFF due to absence of receptors, there is also a positive transcriptional regulation of BAFF production. However, no evidence exists for a direct influence of B cells on BAFF-mRNA levels in monocytes [30]. It is therefore possible that an indirect mechanism is responsible for upregulation of BAFF mRNA levels in monocytes. An important factor for the stimulation of BAFF production is type I IFN [36] , but serum levels of these cytokines decrease rather than increase after RTX treatment. It thus remains to be shown how BAFF-mRNA levels are upregulated after RTX treatment.

APRIL levels are rather stable after B cell depletion with RTX. The two major receptors for APRIL, TACI and BCMA, are largely restricted to activated B cells and to plasma cells, respectively [37, 38]. Since numbers of activated B cells are relatively low, and plasma cells are largely unaffected by RTX treatment, it is therefore not surprising that depletion of peripheral B cells, dominated by mature B cells that do not express appreciable levels of TACI and/or BCMA, does not result in a change in serum APRIL levels.

The observed increase of serum BAFF levels after RTX treatment may result in undesired side effects on selection and survival of autoreactive B cells and on B cell

activation. B cell receptor (BCR) signaling and BAFF-R signaling synergize in B cell survival, which is mediated by activation of NF-kB [39]. BCR signaling activates the classical NF-kB pathway, that results in the production of p100 substrate, required for BAFF-R signaling via the alternative NF-kB pathway [40]. Evidence from studies with transgenic mice indicates that BAFF levels regulate the stringency of the selection of naïve B cells [21, 41, 42]. Anergic B cells compete poorly with non-anergic B cells when BAFF levels are limited and excess of BAFF rescues autoreactive cells that are anergized after the early transitional B cell stage, but not at the early B cell stage [41]. One possible explanation is that autoreactive transitional B cells downregulate their BCR, which results in less availability of substrate (p100), required for BAFF-R signaling. As a consequence these cells require higher BAFF levels for their survival and differentiation to mature B cells [20]. In contrast, long-lived memory B cells, that arise after an antigen driven autoimmune response, appear to be completely independent from BAFF (and APRIL) [18, 20]. The notion that BAFF levels regulate the selection of naïve B cells is based on studies with (genetically-engineered) mice (reviewed in Liu and Davidson [43]), and studies in human are entirely lacking. Nevertheless, higher BAFF levels are speculated to play a role in the pathogenesis of various autoimmune diseases, including pSS [26, 44]. Whether the elevated serum BAFF levels observed after RTX treatment also impair the stringency of the selection of naïve B cells in pSS patients, and therewith promote autoreactivity, is not known. The majority of re-emerging B cells after RTX treatment are transitional type B cells [32] and it remains to be seen that this B cell fraction comprises more autoreactive cells compared to placebo treated patients. In conclusion, we cannot completely rule out the possibility that the elevated BAFF levels contribute to the new formation of autoreactive cells from the bone marrow, but, probably BAFF does not affect the survival of long-lived autoreactive memory cells.

Another effect of the rise in BAFF levels after RTX treatment might be that higher BAFF levels could result in hyperactivation of B cells. Hyperactivation of B cells can be reflected by elevated IgG levels. Others have shown in patients with pSS, RA or SLE that the levels of IgG correlate well with the levels of BAFF. We could, however, not confirm this observation when we looked at baseline serum BAFF and IgG levels. Differences in patient populations and treatment regimes might well explain this discrepancy. Furthermore, after RTX treatment the levels of BAFF rise, whereas IgG levels decline simultaneously. A decrease in IgG levels has also been observed in SLE patients treated with RTX [45]. Thus, clearly after RTX there is no positive correlation between BAFF levels and IgG levels, and there is no evidence that after RTX therapy the higher BAFF levels results in hyperactivation of B cells, persisting in lymphoid organs and tissues. On the other hand, after RTX treatment, IgG levels correlated well with B cell numbers in peripheral blood. A decrease in total B cell numbers due to B cell

depletion therapy could result in an insufficient replacement of long-lived plasma cells. Taken the half-life of IgG into account, fewer plasma cells could result in lower IgG levels. That even severe B cell depletion results in only a slight reduction of IgG in serum might be explained by the fact that most IgG plasma cells are probably long-lived. This is also reflected by our finding that Ig producing cell clones present before therapy, persist up to a year after RTX therapy [46].

Survival of long-lived plasma cells is supported by APRIL by its interaction with BCMA [18], in conjunction with IL-6 and CXCL12/Sdf-1 [47]. These latter two cytokines are found in close proximity to the plasma cells and form special niches in the salivary glands of pSS patients [48]. The high APRIL levels, before and after RTX treatment, may further support the survival of IgG producing plasma cells in the salivary glands of pSS patients. BAFF can also bind to BCMA on the plasma cells. BAFF is produced locally in salivary gland tissue by epithelial cells, T cells and B cells [36, 49, 50]. Ittah et al. [25] showed that the expression of BAFF by salivary gland epithelial cells increases after stimulation with type I IFN or by interaction with viral RNA. This increased expression of BAFF may lead to local activation of autoreactive mature B cells leading to chronic auto-immune inflammation [24, 25]. In our study, we demonstrated the presence of BAFF in parotid gland tissue even after RTX treatment. It can be speculated that this local BAFF is also involved in the survival of local plasma cells after RTX-treatment.

High BAFF levels have been implicated in the pathogenesis and disease activity of pSS [26]. In this study we have shown that BAFF levels further increase after RTX treatment. These elevated BAFF levels likely contribute to repopulation of the naïve B cell pool. Indeed, Pers et al. [51] demonstrated that higher BAFF levels before RTX treatment lead to a shorter period of B cell depletion. This has implications for the development of treatment modalities. It can be speculated that adding anti-BAFF to RTX-treatment may lead to a prolonged reduction of (auto reactive) B cells. Therefore, combining B cell depletion therapy with RTX and anti-BAFF therapy might result in a prolongation of B cell depletion. The additional beneficial effect might be that there is less de novo generation of autoreactive cells after depletion by RTX. Since B cell reappearance correlates well with clinical relapse [6] such a treatment modality might be an appropriate selection.

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CHAPTER 5

Predominantly pro-inflammatory cytokines decrease after B cell depletion therapy in patients with primary Sjögren's syndrome

This chapter is an adapted version of:

Pollard R.P.E., Abdulahad W.H., Bootsma H., Meiners P.M., Spijkervet F.K.L., Huitema M.G., Burgerhof J.G.M., Vissink A., Kroese F.G.M. *Ann Rheum Dis.* 2013, accepted for publication

Abstract

Introduction

The mechanism by which B cell depletion by rituximab (RTX) exerts its beneficial clinical effect in primary Sjögren syndrome (pSS) needs further elucidation. As cytokines play a critical role in the initiation and perpetuation of the chronic inflammatory response in pSS, we explored whether the beneficial effect of RTX on disease activity is mediated by a change in (pro-inflammatory) cytokine levels.

Methods

Thirty pSS patients were treated on days 1 and 15 with either RTX (1,000 mg, n= 20) or placebo (n= 10). Blood samples were collected at baseline and at 5, 12, 36 and 48 weeks after treatment. In addition, blood samples were collected from age and sex matched healthy controls (HC, n=10). B cells were examined by four-color cytometry. A multiplex-bead array cytokine assay was used for simultaneous measurement of 25 cytokines and chemokines.

Results

Depletion of B cells was observed in the RTX group at 5 weeks after treatment, partial reconstitution of B cells started after 12 weeks. At baseline, when compared to HC, all, but IL-8, IFN- γ and RANTES, cytokine levels were increased in patients with pSS. With regard to RTX or placebo treatment, linear mixed effect analysis showed a significant decrease in a subset of, mainly pro-inflammatory, cytokines (GM-CSF, IL-1Ra, IL-6, IL-10, IFN-α, TNF-α) and chemokines (MIP-1β (CCL4), MIG (CXCL9)) in RTX treated patients. Levels of these cytokines and chemokines were significantly decreased, compared to baseline levels, at 5-12 weeks after RTX treatment ($p<0.05$). After this initial decline, levels had reached baseline levels by 36 weeks after RTX treatment.

Conclusion

This study demonstrated that depletion of B cells resulted in a concomitant decrease in serum levels of a subset of mainly pro-inflammatory cytokines. Our findings suggest that the beneficial effect of RTX in pSS patients is mediated by depletion of pro-inflammatory cytokine producing B cells. This insight may contribute to better understanding of the role of B cells in the pathogenesis of pSS and development of more effective drug targeting strategies.

Introduction

Primary Sjögren's syndrome (pSS) is a systemic autoimmune disease characterized by chronic inflammation and destruction of exocrine glands, primarily the salivary and lacrimal glands [1-3]. The main clinical features are progressive dryness of mouth (xerostomia) and eyes (keratoconjunctivitis sicca). General symptoms of pSS include fatigue, weight loss and fever [1]. Extraglandular manifestations may occur at the level of skin, joints, muscles, peripheral and central nervous system, kidneys and lungs [4-7]. These clinical manifestations lead to a diminished health related quality of life in patients with pSS [8].

In the salivary glands, the infiltrates are primarily located around the ducts and are largely composed of T and B lymphocytes, macrophages and (plasmacytoid) dendritic cells [9, 10]. These infiltrates can even be organized to ectopic lymphoid tissues with segregated T and B cell areas, germinal center like structures and high endothelial venules [11].

Cytokines and chemokines play a critical role in the initiation and perpetuation of the chronic inflammatory response in glandular tissue of patients with pSS [12, 13]. A wide variety of both pro- and anti-inflammatory cytokines and chemokines is involved in the inflammatory process, leading to infiltrates and ultimately the destruction of the glands. Important cytokines in this process include interleukin (IL)-6 and tumor necrosis factor (TNF)-α. These cytokines are not only actively synthesized in the salivary glands, but are also overexpressed in saliva and serum of pSS patients [14-16]. Their presence in saliva indicates that they are directly involved in the local inflammatory process [17, 18]. Besides driving inflammation, cytokines can also assert a direct effect on salivary gland function. This is illustrated by the notion that chronic exposure of parotid gland epithelial cells to the pro-inflammatory cytokines TNF-α and interferon (IFN)-γ led to a disruption of tight junction integrity followed by an increase in paracellular permeability of the epithelium [19]. Increased permeability affects the composition of the secreted saliva.

B cells play a critical role in the pathogenesis of pSS and appear to be hyperactivated [1, 20]. Increased activation of B cells in pSS is reflected by the increased levels of serum immunoglobulins (hyper-gammaglobulinemia) amongst which autoantibodies directed against Ro/SSA and La/SSB antigens [20]. The disease is characterized by its type I interferon (IFN) signature, leading to high levels of B cell activating factor (BAFF) in pSS [21]. The high levels of cytokines IL-6 and BAFF may be responsible for B cell hyperreactivity in pSS [22]. The importance of B cells in pSS is further exemplified by the beneficial effect on the clinical symptoms upon administration of rituximab (RTX), a chimeric monoclonal antibody to CD20, leading to peripheral B cell depletion [23-26]. The mechanism by which RTX exerts its beneficial clinical effect remains to be elucidated. In addition to their classical role in antibody formation, B cells also exert

important other immunological functions, such as cytokine production and antigen presentation [27]. Studies indicate that B cells can produce a variety of cytokines including granulocyte-macrophage colony stimulating factor (GM-CSF), IL-6, IL-10, IFN-γ and TNF-α [28-30].

In the work presented here we explored the possibility that the effect of RTX on disease activity in pSS patients is mediated by depletion of cytokine producing B cells. If B cells are indeed a (major) source of cytokine production this might be reflected by decreased cytokine levels in peripheral blood of pSS patients [31]. This insight may contribute to a better understanding of the role of B cells in the pathogenesis of pSS and to more effective drug targeting strategies in this autoimmune disease.

Materials and methods

Patients and controls

Thirty patients with pSS were treated in a randomised double-blinded placebo-controlled trial on days 1 and 15 with intravenous (IV) injections of either 1000 mg RTX (n= 20) or placebo (n= 10) in the University Medical Center Groningen, The Netherlands. To minimize side effects (infusion reactions, serum sickness), all patients were treated with methylprednisolone (100 mg IV), acetaminophen (1000 mg orally) and clemastine (2 mg IV) and received 60 mg oral prednisone on days 1 and 2, 30 mg on days 3 and 4, and 15 mg on day 5 after each infusion. All patients fulfilled the European-US criteria for pSS, as well as the recently published ACR criteria [2, 3]. A detailed account of the patients included in this trial has been described before [23].

From these 30 clinical trial patients, 28 patients (1 male, 27 females; mean age 43 years (range 20-69 years)) were included in the present study. One patient (RTX group) was excluded due to the development of serum sickness like disease and another patient (placebo group) because of insufficient sample at baseline. In addition, 10 age- and sex-matched healthy controls (HC) were included in the current study (1 male and 9 females; mean age 46 years (range 29–60 years)). All patients and controls provided informed consent in accordance with the ethics committee of the University Medical Center Groningen (METC approval: 05.229).

Blood samples

Blood samples were collected at baseline and at 5, 12, 36 and 48 weeks following RTX or placebo treatment. In addition, blood samples were collected from healthy controls. Serum was collected, aliquoted and stored at -20°C until analysis.

B cell analysis

Numbers of circulating CD19+ B cells were quantified by four colour cytometry

using the FACSCalibur flow cytometer and TruCOUNT tubes (Becton Dickinson, Breda, The Netherlands) as previously described [32].

Multiplex cytokine assay

A multiplex-25 bead array cytokine assay (Invitrogen, Breda, The Netherlands) was used enabling simultaneous measurement for the following cytokines and chemokines: GM-CSF, IL-1β, IL-1 receptor antagonist (IL-1Ra), IL-2, IL-2R, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12p40/p70, IL-13, IL-15, IL-17, IFN-α, IFN-γ, TNF-α, monocyte chemotactic protein (MCP-1/CCL2), macrophage inflammatory protein (MIP-1α/CCL3), MIP-1β (CCL4), regulated upon activation normal T cell expressed and secreted (RANTES/CCL5), Eotaxin (CCL11), monokine induced by gamma-interferon (MIG/CXCL9) and interferon-gamma inducing protein (IP-10/CXCL10). Standard curves for each cytokine were generated using the reference cytokine and chemokine concentrations supplied in the kit.

The assay was used in a 96-well plate provided in the kit. All incubation steps were performed at room temperature and in the dark due to the light sensitivity of the bead mix according to manufacturer instructions. In short, the 96-well plate was prewetted with 200 µl of working washing solution; this solution was aspirated using a vacuum manifold. The beads were pipetted into each well and wells were washed two times with washing buffer and were subsequently vacuum filtered. Incubation buffer (50 µl) and 1:4 diluted serum samples or standards (50 µl) were pipetted into the well and incubated for 2 hours with the beads. Thereafter, wells were washed using the vacuum manifold and detector antibody conjugated to biotin (diluted 1:10 with biotin diluent) was added. After incubation for 1 hour, beads were washed followed by an incubation of 30 minutes with streptavidin conjugated to R-phycoerythrin (streptavidin-RPE, diluted 1:10). After washing to remove unbound streptavidin-RPE, beads were analyzed in the Luminex 100 instrument. Raw data (mean fluorescent intensity) from all kits were analyzed by MasterPlex Quantitation Software (MiraiBio, Inc., Alameda, CA, USA) to obtain concentration values.

Statistical analysis

The nonparametric Mann-Whitney U test was used for comparison of cytokine/chemokine values between HC and patients with pSS at baseline. In patients with pSS, a linear mixed effect analysis test (SPSS version 18.0) was used to analyze which cytokines/chemokines were affected by RTX treatment in comparison to placebo treatment during the study period. The linear mixed effect analysis test was performed using time and treatment as explanatory variables and the log transformed cytokine/chemokine values as response. The Wilcoxon matched pairs test was used for intra-individual comparison of pre- and post-treatment values. Data are presented as the median and range. A P-value of <0.05 was considered statistically significant.

Results

B cells started to reappear 12 weeks after RTX treatment

At baseline, B cell numbers were comparable between RTX treated and placebo treated patients. At 5 weeks, a complete depletion of peripheral blood B cells was observed in the RTX treated group. B cells started to reappear from 12 weeks after RTX treatment. In the placebo treated group, a moderate decrease in B cells was observed at 5 weeks post-treatment only, most likely due to the prednisone administration, given to prevent adverse reactions [32].

In patients with pSS most serum cytokine levels were elevated compared to HC

As we show in table 1, baseline serum levels for nearly all cytokines/chemokines analyzed (cytokines: GM-CSF, IL-1β, IL-1Rα, IL-2, IL-2R, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12p40/p70, IL-13, IL-15, IL-17, IFN-α, TNF-α; chemokines: MCP-1 (CCL2), MIP-1α (CCL3), MIP-1β (CCL4), Eotaxin (CCL11), MIG (CXCL9) and IP-10 (CXCL10)) were significantly higher in patients with pSS compared to HC (Mann-Whitney U test, $P<0.05$). Only levels of IL-8, IFN-γ and RANTES (CCL5) did not differ between patients with pSS and HC (Mann-Whitney U test, $P>0.05$).

Mainly pro-inflammatory cytokines and chemokines decreased after RTX treatment

After RTX treatment in patients with pSS, levels of various cytokines and chemokines were decreased. A linear mixed effect analysis showed a significant treatment effect between RTX treated and placebo treated patients for serum levels of the following cytokines: GM-CSF, IL-1Ra, IL-6, IL-10, IFN-α and TNF-α, and the chemokines: MIP-1β (CCL4) and MIG (CXCL9) ($P<0.05$).

In the RTX group, levels of these cytokines and chemokines were significantly decreased, compared to baseline levels, at 5-12 weeks after RTX treatment (Wilcoxon matched pairs test, $P<0.01$) (figure 1A-H). After this initial decline, all followed a gradual return towards baseline levels 36-48 weeks after treatment. Other cytokines (IL-1β, IL-2, IL-2R, IL-4, IL-5, IL-7, IL-12p40/p70, IL-13, IL-15 and IL-17) and chemokines (MCP-1 (CCL2), MIP-1α (CCL3), Eotaxin (CCL11) and IP-10 (CXCL10)) did not differ in their expression levels between the two treatment groups during the studied time period.

In the placebo group, hardly any effects were observed on cytokine/chemokine serum levels. A gradual increase in IL-6 levels was noted in placebo treated patients during the 48 weeks study period. This increase resulted in significantly higher levels of IL-6, 48 weeks after placebo treatment, in comparison to baseline (Wilcoxon matched pairs test, $P<0.05$, figure 1C). Also, in the placebo group MIP-1β (CCL4) serum levels appeared to be higher 36 weeks after treatment compared to baseline (Wilcoxon matched pairs test, $P<0.01$, figure 1G).

Table 1. Serum cytokine and chemokine levels (median (range) values in pg/ml) of 10 healthy controls (HC) and 28 patients with pSS at baseline measured by multiplex-25 bead array cytokine assay

	HC	pSS patients	P-value
Cytokines			
GM-CSF	11.7 (7.2 - 43.0)	315.4 (19.7 - 2517)	0.0001
IL-1β	10.0 (1.9 - 161.1)	411.1 (9.4 - 3103)	<0.0001
IL1-Ra	426.0 (292.7 - 21727)	78864 (742.2 - 1.4 x 106)	<0.0001
IL-2	4.0 (1.2 - 56.5)	154.7 (3.8 - 2152)	<0.0001
IL-2R	114.9 (64.1 - 1265)	3097 (43.4 - 48048)	0.0001
IL-4	31.6 (25.7 - 73.9)	546.5 (32.2 - 11336)	<0.0001
IL-5	2.5 (1.1 - 3.2)	14.5 (2.0 - 458.7)	0.0002
IL-6	4.1 (1.4 - 28.6)	30.2 (2.6 - 332.4)	0.0002
IL-7	17.6 (6.4 - 58.2)	176.7 (8.3 - 1428)	0.0008
IL-8	15.9 (8.7 - 54.9)	25.1 (7.2 - 281.3)	0.1477
IL-10	2.4 (1.4 - 9.6)	225.6 (5.1 - 927.2)	<0.0001
IL-12p40/p70	214.7 (90.7 - 449.7)	1393 (214.8 - 6665)	<0.0001
IL-13	4.9 (1.3 - 95.8)	98.5 (1.8 - 1933)	0.0004
IL-15	18.5 (10.3 - 723.2)	2080 (23.6 - 12284)	<0.0001
IL-17	13.0 (7.1 - 40.6)	469.0 (13.5 - 5455)	<0.0001
IFN-α	29.9 (17.9 - 290.7)	495.6 (69.8 - 3051)	<0.0001
IFN-γ	143.1 (91.8 - 238.5)	152.0 (83.7 - 259.7)	0.85950
TNF-α	3.5 (2.2 - 7.3)	41.05 (4.6 - 1107)	<0.0001
Chemokines			
MCP-1 (CCL2)	636.4 (291.5 - 1209)	1950 (373.7 - 17388)	<0.0001
MIP-1α (CCL3)	83.3 (49.9 -1012)	1520 (92.6 - 7008)	<0.0001
MIP-1β (CCL4)	84.9 (32.8 - 807.3)	1082 (130.7 - 7304)	<0.0001
RANTES (CCL5)	3808 (2652 - 44920	3870 (2791 - 4680)	0.3940
Eotaxin (CCL11)	196.8 (74.4 - 264.0)	412.9 (110.8 - 911.0)	0.0011
MIG (CXCL9)	45.1 (18.6 - 217.8)	1552 (68.6 - 29815)	<0.0001
IP-10 (CXCL10)	77.4 (36.2 - 248.0)	237.1 (23.1 - 1669)	0.0003

Figure 1: Cytokine and chemokine levels

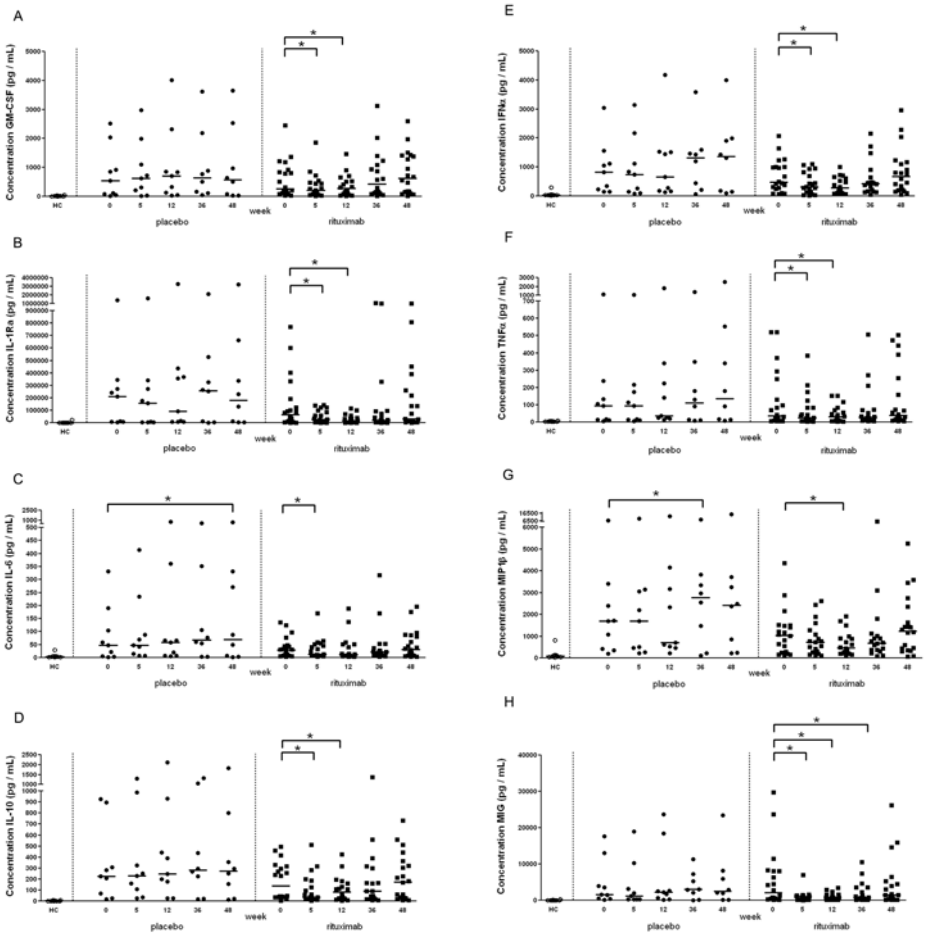

Figure 1. Cytokine and chemokine serum levels of healthy controls (HC) and effect of RTX treatment in pSS patients. The horizontal lines indicate the median values in pg/ml. *indicates significant differences compared with baseline values ($P<0.05$). Only cytokines and chemokines that showed a significant treatment effect of RTX treatment compared to placebo treatment are shown. (A) GM-CSF; (B) IL-1Ra; (C) IL-6; (D) IL-10; (E) IFN-α; (F) TNF-α; (G) MIP-1β (CCL4); (H) MIG (CXCL9)

Discussion

In this study we demonstrated that depletion of B cells resulted in a concomitant decrease in serum levels of a selected number of cytokines (GM-CSF, IL-1Ra, IL-6, IL-10, IFN-α and TNF-α) and chemokines (CCL4 and CXCL9). Most of these cytokines and chemokines belong to the group of pro-inflammatory cytokines. These decreased serum levels of cytokines and chemokines may help explain, at least in part, the positive effects on disease activity after RTX treatment [23, 24, 26].

It is known that local synthesis and secretion of cytokines and chemokines in glandular tissues play a critical role in the inflammatory process of pSS [13, 16]. Many cytokines and chemokines are expressed at higher levels in the salivary and lacrimal glands, indicating that most arms of the immune system are intensely involved in the immunopathogenesis. These cytokines and chemokines are subsequently released in the secretions (saliva and tears) and in serum. In this study we showed that at baseline nearly all cytokines and chemokines analyzed are elevated in serum of patients with pSS, compared to healthy controls. Our observations are in agreement with other studies collectively showing the broad increase of cytokines and chemokines in these patients [14, 15, 33-35].

RTX treatment results in a rapid depletion of CD20 expressing B cells. This is reflected by the virtually complete absence of B cells in peripheral blood 5 weeks after treatment. However, not all B cells are depleted, some cells persist in secondary lymphoid organs and in the affected salivary glands of patients with pSS [36, 37]. In addition, fully matured plasma cells do not express CD20 and are not targeted by RTX. We have already shown, by analysis of variable immunoglobulin (Ig) heavy chain genes, that certain clones of long-lived B cells/plasma cells persist in the salivary glands after RTX treatment [36]. Indeed, serum IgG levels (including autoantibodies) decreased only moderately after one course of RTX treatment in patients with systemic lupus erythematosus (SLE) [38] or pSS [23]. One study in RA showed that IgG levels were not affected at all after RTX treatment [39].

Besides immunoglobulin production, B cells are also responsible for the production of a number of cytokines [28-30]. In line with this notion, we observed in the current study that serum levels of only certain cytokines and chemokines are decreased after RTX treatment, but not after placebo treatment, in pSS patients.

Patients with pSS have a pathologic overproduction of type I IFN (IFN-α, IFN-β) [40, 41] and have a type I IFN signature [21]. The IFN-α inducing capacity of serum of pSS patients is positively associated with labial salivary gland focus scores [40]. Indeed we observed increased levels of IFN-α in our patients at baseline, which decrease after RTX treatment. Plasmacytoid dendritic cells (pDCs) are specialized in the production of type I IFN [41]. Furthermore, it is shown that B cells enhance IFN-α production [42]. B cell depletion could then explain the lower IFN-α serum levels. In addition, also

GM-CSF might play a role in the activation of pDCs [43]. Ghirelli et al. demonstrated that GM-CSF can activate pre-pDCs in a Toll-like receptor (TLR) independent manner [43]. B cells are able to produce GM-CSF, therefore, depletion of B cells not only explains the lower GM-CSF levels after RTX treatment, but may also contribute to lower IFN-α levels [30]. Interestingly, IFN-α was shown to induce production of IL1-Ra and MIG (CXCL9) in patients with SLE [44, 45]. We speculate that lower levels of IFN-α subsequently results in a decrease of IL1-Ra and MIG (CXCL9) in serum of RTX treated patients with pSS.

Besides production of GM-CSF, B cells produce a number of other cytokines including the pro-inflammatory cytokines IL-6 and TNF-α and the anti-inflammatory cytokine IL-10 [28, 29]. The depletion of B cells by RTX treatment in pSS patients may therefore directly result in decreased levels of these cytokines. The decreased IL-6 serum levels that we observed after RTX treatment are in line with findings in RTX treated rheumatoid arthritis (RA) patients [46]. Together with TNF-α, IL-6 is one of the most prominent pro-inflammatory cytokines with a wide variety of effects. At the B cell level, IL-6 stimulates B cell proliferation and differentiation to plasma cells, amongst others via IL-21, which contribute to the B cell hyperreactivity and hypergammaglobulinemia seen in patients with pSS [22]. IL-6 is also involved in pSS pathogenesis of the salivary glands, illustrated by the fact that plasma levels of IL-6 correlates with the degree of lymphocytic infiltrate in the glandular tissue [47]. Barr et al [31] showed with experimental autoimmune encephalomyelitis (EAE) in mice that B cell depletion resulted in amelioration of disease, which was mediated by elimination of IL-6 producing B cells. Similarly in patients with multiple sclerosis, the elevated levels of IL-6 secretion by B cells were normalized after RTX treatment. The authors therefore suggested that the reduced disease severity, observed in patients treated with RTX, was the result of elimination of IL-6 producing B cells. Importantly, in patients with pSS, elevated circulating IL-6 levels are also associated with extraglandular manifestations [47]. Furthermore, IL-6 plays an important role in immunopathology by the generation of Th17 pro-inflammatory lymphocytes [48]. Th17 lymphocytes seem to be involved in the formation of ectopic lymphoid follicles in the salivary glands [49]. These ectopic lymphoid follicles have been suggested to exacerbate disease course and propagate chronic autoimmune inflammation [50].

In addition to a decrease in some pro-inflammatory cytokines, also a decrease in IL-10 levels was observed after RTX treatment of patients with pSS. A similar decrease of IL-10 levels has been observed in RA patients treated with RTX [46]. This might be directly due to eradication of IL-10 producing B-cells. IL-10 is predominantly an anti-inflammatory cytokine and suppresses, amongst others, antigen-presentation capacity of antigen presenting cells. However, in the presence of pro-inflammatory inducing factors, such as IFN-α, IL-10 can display a paradoxical pro-inflammatory effect [51]. This pro-inflammatory property of IL-10 has been demonstrated in other autoimmune

diseases, such as SLE [52, 53]. Administration of an IL-10 antagonist in SLE patients showed improvement of clinical manifestations [54]. Furthermore, in pSS, IL-10 has also been related to salivary gland disease activity and extraglandular manifestations, such as cutaneous vasculitis [33]. Finally, in a transgenic murine model for pSS, IL-10 induced both destruction of glandular tissues through apoptosis and lymphocytic infiltration [55]. As mentioned above, after RTX treatment, however, levels of IFN-α decrease, and IL-10 may acquire a more beneficial, anti-inflammatory function, resulting in improvement of disease parameters.

In conclusion, we have shown that RTX treatment in patients with pSS resulted in a significant decrease in the production of a number of cytokines and chemokines. We suggest that this decrease is explained, at least in part, by a direct removal of (pro-inflammatory) cytokine producing B cells. This may subsequently lead to a decline in levels of other cytokines and chemokines. The decreased levels of cytokines, such as IL-6, may contribute to the improvement of disease progression and various clinical and histopathological parameters as found after RTX treatment of patients with pSS [23, 24]. The results from our study may have implications for the development of new treatment modalities, which are eagerly awaited [56]. Based upon our findings we anticipate that more specific treatment approaches, e.g. blockade of IL-6, might be beneficial for patients with pSS.

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CHAPTER 5

CHAPTER 6

B cell depletion by rituximab does not affect frequency and function of regulatory T-cells in patients with primary Sjögren's syndrome

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B cell depletion therapy with anti-human CD20 (rituximab; RTX) has been shown to be an effective treatment strategy for patients with primary Sjögren's syndrome (pSS) [1;2]. However, the mechanisms through which RTX exerts its effects have not been fully elucidated. Besides the immediate effect caused by destruction of autoreactive B cells, RTX-therapy may also influence the frequency and function of regulatory T cells (Tregs). Previous studies have shown that RTX-treatment restored the impaired Tregs of patients with systemic lupus erythematosus (SLE) or idiopathic thrombocytopenic purpura (ITP) [3-5]. Therefore, augmentation of Tregs mediated effects on peripheral self-tolerance after RTX-therapy may be an additional pathway by which such a therapy is effective in patients with autoimmune diseases.

In the work described here we analyzed the frequency of Tregs in parotid gland biopsies before and 12 weeks after RTX (20 patients) and placebo (10 patients) treatment . A detailed account of the patients included in this trial has been described before [2]. In addition the proportion and function of Tregs cells in freshly isolated PBMCs from 5 pSS-patients, at baseline and at 24 weeks after RTX-infusion, and from 5 age- and sex-matched healthy controls was assessed.

The frequency of Tregs in parotid gland was assessed by double staining with CD3 and FOXP3. The proportion of Tregs was assessed by flow cytometry using CD4, CD25 and FoxP3 markers, whereas suppressive function of sorted Tregs was determined by co-culture assay.

No difference in the amount of Tregs in the parotid gland was observed after placebo ($p= 0.6523$) or RTX ($p=0.4212$) treatment compared to baseline (figure 1 and 2). Furthermore, we showed that RTX-treatment of pSS-patients did not alter the frequency (figure 3) and function (figure 4) of Tregs cells. This different outcome of RTX-therapy in pSS-patients, compared to patients with SLE and ITP, is not completely clear. A likely explanation for this difference might be due to the fact that baseline levels of Tregs-function in pSS-patients are not impaired (figure 4), in contrast to Tregs in patients with SLE and ITP. We suggested that these (normal) levels of Tregs numbers and function may not allow further upregulation in frequency and function of Tregs in pSS-patients after RTX-treatment. The finding that RTX-therapy affects Tregs in patients with SLE and ITP argues that B cells directly contribute in the abrogation of Tregs function. It has been speculated by Vigna-Perez el al. [4] that depletion of B cells in SLE-patients would induce a decrease in activated T cells expressing glucocorticoid-induced TNF-receptor ligand (GITRL), a ligand that is able to impair the frequency and function of Tregs [6;7]. Therefore, it is feasible that a decrease in GITRL+ cells after RTX-treatment favour an increase in number/function of Tregs in SLE- and ITP-patients. However, data regarding the impact of B cell depletion on GITRL+ cells in relation to Tregs properties in pSS-patients are lacking. Obviously, further sophisticated studies in pSS are needed to address this speculation. On the other hand, RTX-therapy in pSS-patients induce a dramatical reduction in serum IL-10 [8], an essential cytokine for

Tregs generation [9]. The decrease in IL-10, therefore, may prevent the expansion of Tregs in pSS-patients. In general, it seemed that the observed positive effects of RTX-treatment in pSS-patients did not act through the Tregs arm.

In summary, we show that Tregs frequency in parotid tissue are unaffected and that both the relative frequency and the suppressive function of Tregs from pSS-patients at baseline are not impaired, and remained unaffected during B cell depletion following RTX-treatment. Thus, the effectiveness of RTX-treatment in pSS-patients is not associated with enhancement of Tregs numbers and/or function.

Figure 1A: Parotid biopsy specimen before RTX treatment

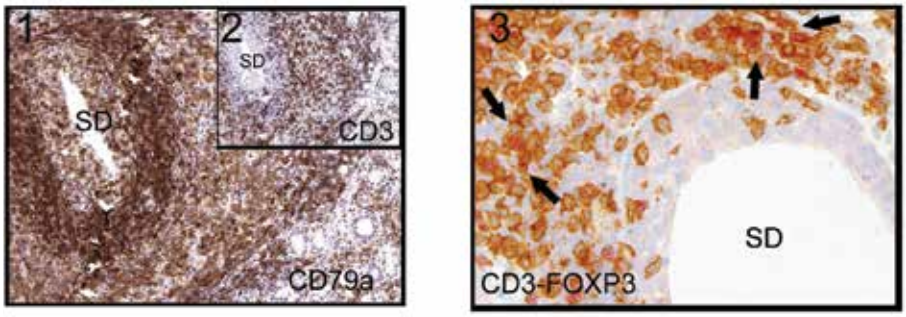
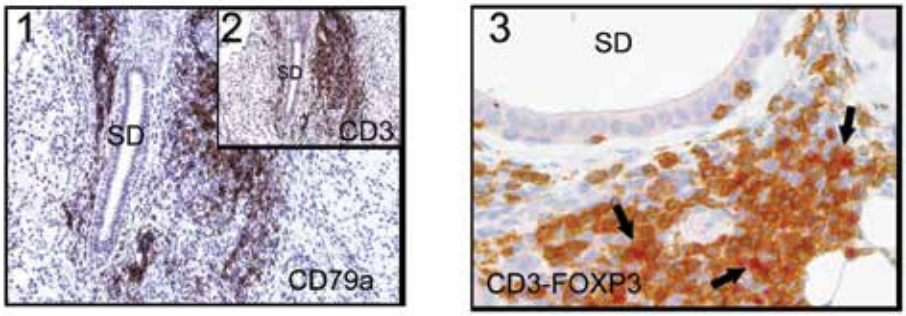


Figure 1B: Parotid biopsy specimen 12 weeks after RTX treatment



A. Immunohistochemistry for CD79a B-cells (1A-1, brown) and CD3 T-cells (1A-2, brown) before RTX in parotid gland tissue. Lymphocytic infiltration was mostly located around the striated duct (SD) and consisted of CD3 T-cells and CD79a positive B-cells. CD3-FOXP3 is predominantly localized in the lymphocytic infiltrate (arrows).
B. Immunohistochemistry for CD79a B-cells (1B-1, brown) and CD3 T-cells (1B-2, brown) localization (3B-3, brown) after RTX in parotid gland tissue. A reduction in lymphocytic infiltration around the striated duct (SD) was visible after RTX. CD3-FOXP3 is predominantly localized in the lymphocytic infiltrate (arrows).

Figure 2: Tregs in parotid glands before and after treatment

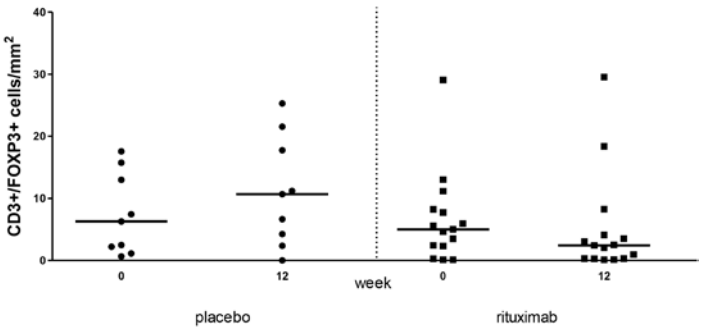


Figure 2. Amount of Tregs in parotid glands of placebo (n=9) or RTX treated (n=15) patients. An incision biopsy was taken under local anesthesia from the same parotid gland before and 12 weeks after placebo (n=10) or RTX (n=20) therapy. No difference in Tregs frequency was observed in placebo (p= 0.6523) or RTX (p=0.4212) treatment group when compared to baseline.

Figure 3: Frequency of circulating Tregs in pSS-patients before and after RTX-treatment, compared with HCs.

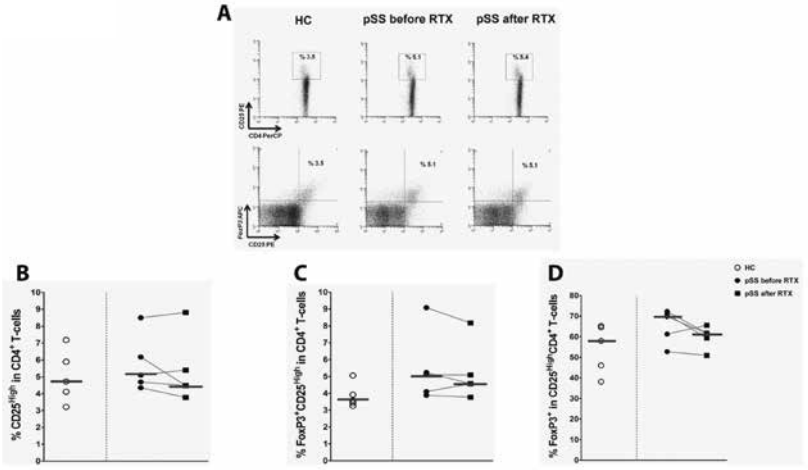


Figure 3. (A) Representative example of FACS-plots and gating of CD25High and FoxP3+CD25High cells among CD4+T-cells in a pSS-patient (middle and right panels) and an age- and sex-matched HC (left panels). Values in each gate represent the percentage of positive cells. (B) Percentage of CD25High – and (C) FoxP3+CD25High cells among CD4+T-cells, and (D) percentage of FoxP3-expression within the CD25HighCD4+T-cells in peripheral blood of pSS-patients (before and after RTX-treatment) and sex- and age-matched HCs. Bars represent median percentage.
In this study, PBMCs from 5 pSS-patients (at baseline and at 24 weeks post RTX-treatment) and 5 age- and sex-matched healthy controls (HC) were isolated immediately from freshly drawn blood by density-gradient centrifugation on Lymphoprep. Cells recovered from the gradient interface were washed and immediately incubated with appropriate concentrations of FITC-anti-CD3, PerCP-anti-CD4, and PE-anti-CD25, for 30 minutes at 4 oC in the dark. Cells were fixed/permeabilized followed by the addition of APC-conjugated rat anti-human-FoxP3. After incubation for 30 minutes at 4 oC, the cell suspension was washed and immediately analyzed on FACS-Calibur (Becton & Dickinson). For all flow cytometry analyses, data were collected for 105 cells. Lymphocytes were gated by forward and side scatter, and plotted using the Win-List software package (Verity Software House Inc, ME, USA). Positively and negatively stained populations were calculated by quadrant dot plot analysis, as determined by the appropriate isotype controls.

Figure 4: Functional characterization of Tregs in pSS-patients, before and after B cell depletion, compared to HCs.

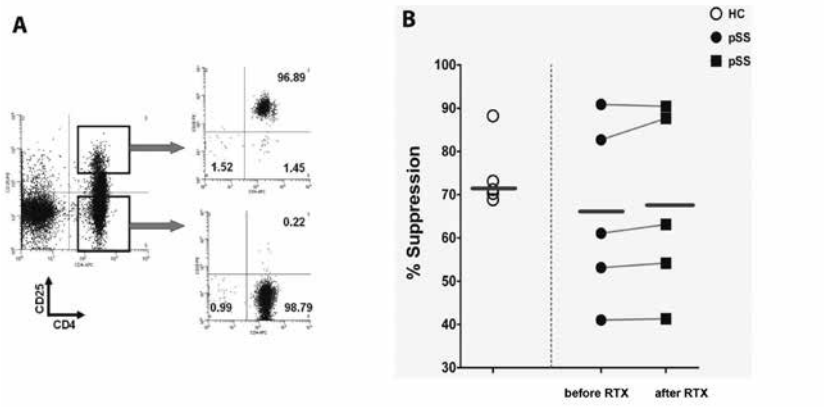


Figure 4. PBMCs from 5 pSS-patients (at baseline and at 24 weeks post RTX-treatment) and 5 age- and sex-matched healthy controls (HC) were isolated immediately from freshly drawn blood and incubated for 45 minutes at 4 °C with appropriate concentrations of APC-anti-CD4, PE-anti-CD25, and a cocktail of anti-CD8, anti-CD56, and anti-γδTCR, all FITC labeled. Cells were washed and sorted on a FACS-MoFlow (Becton & Dickinson) according to their forward and side scatter properties. Subsequently, FITC-positive cells were excluded, and the $CD4^+$T-cell fraction was sorted into a $CD4^+CD25^-$ population as responder T-cells (T_{Resp}) and a $CD4^+CD25^{High}$ population as regulatory T-cells (Tregs). The purity of the sorted T_{Resp} and Tregs, determined by flow-cytometric reanalysis, was >98% and >96%, respectively (**A**).

Freshly sorted T_{Resp} ($1x10^4$ cells per well) and Tregs ($1x10^4$ cells per well) were cultured in triplicate, separately or in co-culture (ratio 1:1), in round-bottom 96-well plates in 200μL of complete RPMI1640 medium. Cells were incubated in the absence or presence of soluble anti-CD3 and anti-CD28 for 6 days at 37°C in 5% CO_2. For the last 18 hours of culture 1 μCi/well (^{3}H)-thymidine was added, and (^{3}H)-thymidine incorporation was counted and expressed as counts per minute. The percentage suppression of proliferation was calculated as follows:

% Suppression= 1- (mean cpm of co-culture / mean cpm of responder cells alone) x 100%

Percentage suppression of Tregs proliferation by autologous Tregs from HCs (n=5), and pSS-patients (n=5) before and after RTX-treatment are shown in figure **B**.

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CHAPTER 6

CHAPTER 7

Treatment of mucosa-associated lymphoid tissue Lymphoma in sjögren's syndrome: A retrospective clinical study

Pollard R.P.E., Pijpe J., Bootsma H., Spijkervet F.K.L., Kluin Ph.M., Roodenburg J.L.N., Kallenberg C.G.M., Vissink A., van Imhoff G.W. *J Rheumatol.* 2011; 10: 2198-208

Abstract

Objective

To retrospectively analyze the clinical course of patients with mucosa-associated lymphoid tissue (MALT)-type lymphoma of the parotid gland and associated Sjögren's syndrome (SS).

Methods

All consecutive patients with SS and MALT lymphoma (MALT-SS) diagnosed in the University Medical Center Groningen, The Netherlands, between January 1997 and January 2009 were analyzed. Clinical course and treatment outcome of SS and MALT lymphoma were evaluated.

Results

From a total of 329 SS patients, 35 MALT-SS patients were identified, with a median follow-up of 76 months (range 16-153 months). MALT lymphoma was localized in the parotid gland in all cases. Treatment consisted of "watchful waiting" (n=10), surgery (n=3), radiotherapy (n=1), surgery combined with radiotherapy (n=2), rituximab only (n=13), or rituximab combined with chemotherapy (n=6). Complete response was observed in 14 patients, partial response in one patient and stable disease in 20 patients.

In six of 7 patients with initially high SS disease activity (M-protein, cryoglobulins, IgM-Rf > 100 KIU/l, severe extraglandular manifestations) their MALT lymphoma progressed and/or SS disease activity increased after a median follow-up of 39 months (range 4-98 months), necessitating retreatment. Only one MALT patient with low SS disease activity showed lymphoma progression when left untreated.

Conclusion

An initially high SS disease activity likely constitutes an adverse prognostic factor for progression of lymphoma and/ or SS. Such patients may require treatment for both MALT lymphoma and SS. In SS patients with localized asymptomatic MALT lymphoma and low SS disease activity, a "watchful waiting" strategy seems justified.

Introduction

Sjögren's syndrome (SS) is a systemic autoimmune disease characterized by chronic inflammation of the salivary and lacrimal glands. SS is frequently accompanied by systemic symptoms. Four-to-seven percent [1;2] of patients with SS develop malignant B cell lymphoma, 48-75% of which are of the mucosa-associated lymphoid tissue (MALT)-type. These B cell lymphomas are most frequently located in the parotid gland [3-5]. A recent study showed a 6.6-fold increase of non-Hodgkin lymphoma (NHL) in SS patients as compared to controls [6]. MALT lymphoma of the parotid gland was almost exclusively associated with SS, as there was a 1000-fold increase in the relative risk of having SS in case of a MALT lymphoma presenting in the parotid gland [6]. In SS patients parotid gland enlargement is frequently present but varies in time. The change from variable to persistent enlargement of glands is an important clinical sign, indicating the possible development of MALT lymphoma. Furthermore, the emergence of lymphoma in SS may be heralded by extraglandular manifestations of SS (e.g., palpable purpura, vasculitis, renal involvement, peripheral neuropathy). None of these features are specific for MALT lymphoma in SS, but any of them should raise suspicion, particularly if accompanied by features such as monoclonal gammopathy, reduced levels of complement C4, CD4+ T lymphocytopenia, a sharp decrease in IgG levels or cryoglobulinemia [2;7-10].

Assessment of SS patients who may have developed a MALT lymphoma is not always easy, but an incisional biopsy of the parotid gland can safely be performed under local anesthesia [11].

MALT lymphoma in general is an indolent disease, with a reported 5-year overall survival between 86% and 95%, without significant difference in clinical course between localized and disseminated disease [12;13]. Recurrences may involve extra-nodal or nodal sites. Progression to aggressive lymphoma is rare, occurring in less than 10% of cases [14].

MALT lymphoma in patients with SS is often localized at one or more salivary glands [usually the parotid gland[s]], but can also occur in other extranodal sites, such as the orbital adnexa [15] and stomach [16]. Dissemination of MALT-SS may be observed in local draining lymph nodes or, sometimes, distant nodes, and, occasionally, other mucosal sites and bone marrow [17]. Prognosis does not seem to be influenced by dissemination to other MALT organs, although involvement of lymph nodes might be an adverse prognostic factor [13;17].

The relative infrequency and heterogeneity of MALT lymphomas, with their different biology, clinical presentation and behaviour, makes it difficult to define optimal treatment of these patients in general. Antibiotics are widely used as initial treatment of MALT lymphoma associated with microbial pathogens, in particular gastric MALT

lymphoma associated with Helicobacter Pylori [18-21]. In other MALT lymphomas with symptomatic local disease, local treatment (surgery or radiotherapy) results in excellent disease control [22]. However, in SS patients conventional radiotherapy of the salivary glands (25 to 39 Gy) may lead to significant further xerostomia [23;24]. An alternative approach might be low-dose 2x2 Gy involved field radiotherapy. This therapy is very effective in follicular lymphoma [25] and data in MALT lymphoma seems promising [26;27]. For symptomatic disseminated disease, chemotherapy has commonly been used, with 75% complete remission rate and 5 years event-free survival and overall survival rates of 50% and 75% respectively [12;13;28-30]. More recently, Rituximab, a chimeric murine/human anti-CD20 monoclonal antibody that is highly efficacious in patients with B cell lymphoma, alone or in combination with chemotherapy [31-34], has also been used effectively in patients with MALT lymphoma, with or without associated SS [35-40].

At present, no clear guidelines exist for the management of patients with MALT-SS. In this retrospective study we report our experience in 35 consecutive patients with MALT-SS treated in our center.

Patients and methods

Patients

Our hospital, the University Medical Center Groningen, is a referral institute for SS in the Netherlands. Patients are referred to either the department of Rheumatology or Oral Medicine. Subsequently, all patients with suspicion of SS are routinely evaluated in both departments. In case of MALT lymphoma, patients are also seen by a hematologist. A retrospective analysis was performed of all consecutive patients diagnosed with SS and MALT lymphoma in our hospital, between January 1997 and January 2009. Included were patients with a diagnosis of extranodal marginal zone (MALT) lymphoma according to the WHO classification [41] and a concomitant diagnosis of SS according to the American-European consensus criteria [42]. All MALT-SS patients were seen at 6-month intervals for routine follow-up. Follow up ended on February 1, 2010 (median follow-up 76 months, minimal follow-up of 12 months).

Diagnosis of SS and MALT lymphoma

A full routine diagnostic work-up for SS was carried out in all patients at their first visit as described before [38]. In short, this work-up included: subjective complaints of ocular and oral dryness, eye tests (Rose bengal staining and Schirmer tear test), measuring unstimulated whole salivary flow, parotid sialography, and serology (anti-SSA/B-antibodies). In all patients an incision biopsy of the parotid gland was part of the SS work-up and performed under local anesthesia [11;43]. All patients in our cohort were diagnosed for SS using the SS classification criteria as stated by the

American-European SS association, Hepatitis C and HIV infections were excluded accordingly [42].

All biopsies were reviewed by a hematopathologist (PK). In all 35 cases immunohistochemistry was performed for at least CD3, CD5, CD20, CD79a, CD10, BCL6 and cytoplasmic immunoglobulins [kappa, lambda, IgM, IgG and IgA]. In 21/35 cases DNA analysis of clonality was performed using PCR for FR1, FR2 and FR3 of the immunoglobulin heavy chain gene complex, as described by Van Dongen et al.[44] and Sandberg et al.[45] All FR PCR reactions were performed in fourfold at two dilutions. All 21 cases revealed a dominant and reproducible monoclonal population of B cells in one or more framework PCR reactions. To distinguish between reactive benign lymphoproliferation and MALT lymphoma, the classification of Quintana et al. [46] was used. All lymphomas were classified according to the WHO classification published in 2008 [41].

Staging of MALT lymphoma and disease activity score of SS

Patients were staged according to a standard lymphoma protocol including CT or MRI-scans of head/neck, thorax and abdomen, and a bone marrow biopsy [47]. For SS associated MALT lymphomas located in the salivary gland, we used a relatively simple staging system based on the Ann Arbor classification [47] and the modification for primary gastric lymphoma by Musshoff [48] as follows:

(1) Localized disease: lymphoma located in one or more salivary glands [unilateral or bilateral], without enlargement of lymph nodes;
(2) Locally disseminated disease: lymphoma localized in one or more salivary glands [unilateral or bilateral] with one or more enlarged regional lymph nodes [> 1 cm];
(3) Disseminated disease: localization of lymphoma in one or more salivary glands [unilateral or bilateral] with one or more enlarged distant lymph nodes (> 1 cm), and/or bone marrow, spleen, liver, or other extranodal site than the salivary gland, or localization of lymphoma in multiple extranodal sites.

SS disease activity was evaluated based on the following variables: the presence of extraglandular manifestations (e.g. arthritis, fatigue, vasculitis, glomerulonephritis), subjective sicca symptoms (using a 100-mm visual analogue scale (VAS)), salivary gland function and serological parameters (levels of total IgG and IgM rheumatoid factor (IgM-Rf), C4, cryoglobulins, M-protein). Based on these parameters a global impression of disease activity (low, moderate, high) was determined by the physicians (R.P., J.P., H.B., F.S., G.v.l.) participating in the multidisciplinary group for patients with SS and MALT lymphoma.

Treatment and treatment evaluation

The choice of treatment modality was decided by a team of experts (rheumatologist, oral surgeon, pathologist, haematologist) based on clinical, serological and radiographic data. In general the following treatment regimens were used: watchful waiting, surgery, radiotherapy, surgery combined with radiotherapy or cyclophosphamide-prednisone. Rituximab was available from 2002 onwards and added to the treatment regimens. Rituximab monotherapy was given as 4 weekly infusions of 375 mg/m2 [38]; Rituximab with cyclophosphamide and prednisone (R-CP) was given as 6-8 intravenous infusions of 375 mg/m2 of rituximab and 750 mg/m2 cyclophosphamide. One infusion was given every 3-4 weeks, in combination with 100 mg prednisone p.o. for 5 days. After initial rituximab or R-CP treatment no maintenance immunotherapy was given.

Time between diagnosis of SS and presentation of lymphoma, response to treatment, and clinical course during follow-up were retrieved from the medical records. Tumor responses were classified as complete response (CR), partial response (PR), stable disease (SD), or recurring/progressive disease (PD), according to the standardized response criteria for malignant lymphomas [49;50]. In brief, CR required the absence of palpable swelling of salivary glands and return of all nodes to less than 1 cm on CT/MRI, and normalization of bone marrow, spleen, liver or other extranodal sites, if initially involved. In patients with localized disease CR was considered when no evidence of disease was present after diagnostic surgical excision with or without subsequent radiotherapy or after R-CP objectified by MRI. Seven patients treated with rituximab monotherapy took part in a prospective clinical trial in which a repeated parotid gland biopsy was performed after treatment as defined by protocol [38]. In these patients, a repeated biopsy of the involved parotid gland had to show complete disappearance of the lymphoma infiltrate for classifying these patients to have reached CR. PR required regression of initial tumor mass by ≥ 50% without development of new lesions. SD was defined as < 50% regression or < 50% increase of the known sites of disease. PD recurrence required ≥ 50% increase of any previously identified abnormal lesions or any new lesion (including recurrence in case of previous CR), irrespective of simultaneous responses at other sites.

Since there are no validated disease activity response criteria for SS yet [51;52], deterioration of SS was arbitrarily defined as the occurrence of one or more of the following: an increase in levels of IgG, an increase in levels of IgM-Rf, decrease in salivary gland function, increase in subjective oral or ocular symptoms, and/or the development of extraglandular manifestations [arthritis, vasculitis; pulmonary, hepatic or renal SS involvement]. Improvement of SS required one or more of the following: a decrease in levels of IgG and/or IgM-Rf, improvement of salivary gland function [53], improvement of subjective symptoms, and/or the disappearance of extraglandular manifestations. Stable disease was defined as the absence of deterioration or improvement according

to the above mentioned criteria. The criteria mentioned above are in agreement with the outcome criteria in SS for clinical trials as proposed by Pillemer et al.[54] and the later developed, not yet validated, disease activity scales [51;52].

Results

Patient characteristics

From a total of 329 SS patients diagnosed in our hospital between January 1997 and January 2009, 35 (11%) MALT-SS patients were identified. Two SS patients developed extraglandular lymphoproliferative malignancies without parotid gland involvement; one patient had an inguinal extra-ossal plasmacytoma, the other a rectal MALT lymphoma. For the purpose of this study, these patients were both excluded from analysis. Characteristics of MALT lymphoma and SS of the 35 patients included in this study are listed in tables 1 and 2.

Table 1. Clinical characteristics of the 35 patients with MALT-SS

Characteristic	No. of patients	Percentage (%)
Gender		
male	3	9
female	32	91
Staging MALT*		
Localized disease	26	74
Locally disseminated	5	14
Disseminated disease	4	11
SS disease activity*		
low activity	28	80
high	7	20

*See methods for MALT-SS staging and SS disease activity definitions

In 11 out of 35 (31%) patients MALT lymphoma was detected by chance in a parotid biopsy during routine diagnostic work-up of SS. Eighteen (51%) MALT lymphomas were detected in patients presenting with active SS and suspicion of MALT lymphoma development based on persistent glandular swelling, confirmed after parotid biopsy. Six (17%) patients presented with a parotid gland tumor initially, in whom a diagnosis of MALT lymphoma in association with SS was made following the parotid biopsy.
Median age at MALT lymphoma diagnosis was 55 years (range 26-84). MALT lymphoma was localized in the parotid gland in all cases (n=35). The majority of patients had localized disease (26 out of 35 (74%)) Five patients (14%) had locally disseminated disease, and 4 (11%) patients had disseminated disease (bone marrow in one patient,

Table 2. SS characteristics of the MALT-SS patients

UPN	Age#/ Sex	SS year of diagnosis	MALT year of diagnosis	Risk factors	Extraglandular manifestations	Anti-SSA/SSB positivity	IgG, g/l	IgM-Rf, KIU/l
1*	68/F	1997	1997	PGS, M-protein	Arthralgia, arthritis	SSB	12.6	445
2	77/F	1998	1998	-	Arthralgia, fatigue	-	16.3	860
3	61/F	1998	1998	PGS, M-protein	None	SSA	10.1	<11
4	33/M	1992	1999	PGS	Arthritis, fatigue	SSA/SSB	22.0	107
5	55/F	1999	1999	-	Arthralgia, arthritis	SSA	16.5	95
6	28/F	1990	2001	PGS, low C4	Arthralgia, arthritis, Raynaud's phenomenon	SSA/SSB	24.0	80
7	64/F	2001	2001	PGS, low C4	Arthralgia, arthritis, fatigue	SSA/SSB	10.3	NA
8*	36/F	1998	2002	PGS, purpura, low C4, M-protein, cryoglobulins	Arthralgia, fatigue, Raynaud's phenomenon, vasculitis	SSA/SSB	19.6	461
9	62/M	2003	2003	-	Arthralgia, fatigue	SSA/SSB	22.1	267
10*	54/F	1990	1997	PGS, cryoglobulins	Fatigue, vasculitis, pulmonary, hepatic and renal involvement	SSA	6.8	101
11	48/F	1998	1998	PGS	None	SSA/SSB	12.4	80
12	72/F	2003	2003	PGS	Arthralgia, fatigue	SSA/SSB	14.8	98
13*	50/F	1995	2004	PGS, low C4,M-protein	Arthritis, fatigue, Raynaud's phenomenon, vasculitis and esophageal involvement	SSA/SSB	5.9	342
14*	43/F	2000	2004	PGS, M-protein	Arthritis, fatigue, Raynaud's phenomenon	SSA/SSB	14.7	124
15	57/F	2001	2004	PGS	Fatigue	SSA	15.0	30
16	43/F	1990	2002	-	Fatigue	SSA/SSB	25.0	107
17	76/F	2003	2003	M-protein, low C4	Arthralgia, Raynaud's phenomenon	-	15.5	399
18	58/F	2004	2004	-	Fatigue	SSA	15.1	113
19	36/M	2004	2004	PGS	Fatigue	SSA/SSB	17.4	26
20	57/F	1989	2004	PGS	Fatigue	SSA/SSB	23.3	88
21	67/F	2005	2005	PGS	Fatigue, Raynaud's phenomenon	SSA/SSB	17.3	136
22	51/F	2005	2005	Low C4	Arthralgia, fatigue	SSA/SSB	38.5	278
23	57/F	2000	2003	PGS, normal C4	Fatigue	SSA/B	25.9	96
24	68/F	1990	2003	PGS	Arthralgia	SSA	23.3	440
25	41/F	2005	2005	PGS	Arthralgia, fatigue	SSA/B	18.9	152
26	65/F	2000	2006	PGS	Fatigue	SSA	18	1860
27	72/F	2006	2006	PGS	Fatigue, Raynaud's phenomenon	SSA	14.1	510
28*	64/F	2001	2005	PGS, low C4, M-protein, cryoglobulins	Fatigue, vasculitis, pulmonary and esophageal involvement, polyneuropathy	SSA	19.4	2370
29*	76/F	1992	2006	PGS, low C4, M-protein, cryoglobulins	Arthritis, Raynaud's phenomenon	SSA	10.6	172
30	65/F	2004	2005	PGS	Arthralgia	SSA	12.4	93
31	60/F	2007	2007			-	11,2	19
32	85/F	2007	2007	PGS, low C4,	Fatigue	SSA/SSB	15.0	20
33	42/F	2005	2007	PGS, low C4		-	12,9	9
34	54/F	2006	2007	PGS	Arthralgia	SSA/SSB	13	41
35	37/F	2005	2008	PGS		SSA	13,1	183

Abbreviatons: UPN: unique patient number; *patient with high SS disease activity; see Patients and Methods; #Age at time of diagnosis of MALT-SS; F: female; M: male; SS: Sjögren's syndrome; PGS: parotid gland swelling; M-protein: monoclonal protein present; IgG: immunoglobulin G (normal 8.5-15 g/liter); IgM-Rf: IgM rheumatoid factor (normal <11 kIU/l)

lacrimal gland involvement in two patients, and involvement of the stomach in one patient). Seven patients had high SS disease activity initially, as exemplified by monoclonal gammopathy / cryoglobulins, increased IgM-Rf and one or more severe extraglandular manifestations (arthritis, vasculitis; pulmonary, hepatic or renal SS involvement).

Treatment and response of MALT lymphoma

Ten patients (28%) received no initial treatment because the lymphoma was asymptomatic. These patients were closely monitored ("watchful waiting") (tables 3 and 4). In 5 patients a diagnostic superficial parotidectomy was performed because of persistent symptomatic unilateral parotid gland swelling, which resulted in complete excision of the lymphoma in 3. Three patients received radiotherapy including the 2 patients with incomplete excision of the lymphoma. Thirteen patients (37%) were treated with rituximab only, seven of those patients participated in a phase II study with rituximab and have been described in a previous report. [38] Six patients (17%) were treated with R-CP.

Table 3. Treatment and outcome of the 35 MALT-SS patients

Treatment	N	Stage	Initial high SS disease activity	Total	Outcome		
					CR	PR	SD
Watchful waiting	10	L	0	9	0	0	9
		LD	1	1	0	0	1
		DD	0	0	0	0	0
Surgery	3	L	0	1	1	0	0
		LD	0	1	0	0	1
		DD	0	1	0	0	1
Radiotherapy	1	LD	1	1	0	0	1
Surgery/Radiotherapy	2	L	0	1	1	0	0
		LD	0	0	0	0	0
		DD	0	1	1	0	0
Rituximab	13	L	1	11	3	1	7
		LD	2	2	2	0	0
		DD	0	0	0	0	0
R-CP	6	L	0	4	4	0	0
		LD	0	0	0	0	0
		DD	2	2	2	0	0
Total	35		7	35	14	1	20

Abbreviations: L, localized disease; LD, locally disseminated disease; DD, disseminated disease; CR, complete remission; PR, partial response; SD, stable disease ; R-CP: rituximab-cyclophosphamide-prednisone combination (see patients and methods)

Lymphoma response in the 25 patients treated with surgery, radiotherapy, rituximab or R-CP was as follows: CR in 14 patients (56%), PR in one patient (4%) and SD in 10 (40%) patients. No serious side-effects were observed.

Table 4. Characteristics of MALT-SS patients regarding MALT lymphoma, treatment and outcome

UPN	#Localisation	Treatment	MALT-response after 12 weeks	Progression/ time to progression (months)		Retreatment	Response	Survival/ months
				Lymphoma	SS			
1*	L	Watchful Waiting	SD	Yes/ 81	No	Surgery	CR	Alive/ 153
2	L	Watchful Waiting	SD	No	No			##Deceased/ 109
3	DD	Surgery	SD	No	No			Alive/ 141
4	L	Surgery	CR	Yes/ 85	No	RCP	CR	Alive/ 124
5	L	Watchful Waiting	SD	No	No			Alive/ 128
6	L	Surgery & Radiotherapy	CR	No	No			Alive/ 102
7	L	Watchful Waiting	SD	No	No			Alive/ 108
8*	LD	Rituximab	CR	No	Yes/ 27	Cyclophosphamide	Stable	Alive/ 91
9	L	Rituximab	SD	No	No			Alive/ 78
10*	LD	Radiotherapy	SD	Yes/ 98	Yes/ 98	Cyclophosphamide	Stable	Alive/ 153
11	L	Rituximab	CR	No	No			Alive/ 141
12	L	Rituximab	CR	No	No			Alive/ 75
13*	L	Rituximab	SD	Yes/ 4	Yes/ 4	Rituximab	Stable	Alive/ 66
14*	LD	Rituximab	CR	Yes/ 52	No	Rituximab	Stable	Alive/ 70
15	LD	Surgery	SD	Yes/ 73	No	Radiotherapy	CR	Alive/ 100
16	L	Rituximab	CR	No	No			Alive/ 94
17	L	Rituximab	SD	No	No			Alive/ 83
18	L	Rituximab	SD	No	No			Alive/ 64
19	L	Rituximab	SD	No	No			Alive/ 67
20	L	Watchful Waiting	SD	No	No			Alive/ 64
21	L	Rituximab	PR	Yes/ 9	No	Rituximab	Stable	Alive/ 57
22	L	Rituximab	SD	No	No			Alive/ 58
23	DD	Surgery & Radiotherapy	CR	Yes/ 39	No	RCP	Stable	Alive/ 85
24	L	Watchful Waiting	SD	No	No			Alive/ 75
25	LD	Watchful Waiting	SD	No	No			Alive/ 53
26	L	R-CP	CR	No	No			Alive/ 49
27	L	Watchful Waiting	SD	No	No			Alive/ 46
28*	DD	R-CP	CR	No	No			Alive/ 50
29*	DD	R-CP	CR	Yes/ 15	No	Radiotherapy	CR	Alive/ 47
30	L	Watchful Waiting	SD	Yes/ 34	No	Radiotherapy	CR	Alive/ 50
31	L	R-CP	CR	No	No			Alive/ 36
32	L	R-CP	CR	No	No			Alive/ 28
33	L	Watchful Waiting	SD	No	No			Alive/ 28
34	L	R-CP	CR	No	No			Alive/ 26
35	L	Rituximab	SD	No	No			Alive/ 16

Abbreviatons: UPN: unique patient number;*patient with high SS disease activity; #L: localized disease, LD: locally disseminated; DD: disseminated disease; see Patients and Methods; CR: complete response; SD: stable disease; PR: partial response; RCP: rituximab/cyclophosphamide/prednisone. ## Patient died of pneumonia unrelated to MALT lymphoma.

Table 5. Adverse factors before and after initial treatment of the MALT-SS patients

UPN	Adverse factors				Progression/ Recurrence
	Serologic parameters before treatment	Extraglandular manifestations before treatment	Serologic parameters after treament	Extraglandular manifestations after treatment	
1*	PGS, M-protein	Arthralgia, arthritis	NA	NA	MALT-lymphoma
2	-	Arthralgia, fatigue	NA	NA	
3	PGS, M-protein	None	M-protein	None	
4	PGS	Arthritis, fatigue	-	Arthritis	MALT-lymphoma
5	-	Arthralgia, arthritis	NA	NA	
6	PGS, low C4	Arthralgia, arthritis, Raynaud's phenomenon	low C4	Arthralgia, arthritis, Raynaud's phenomenon	
7	PGS, low C4	Arthralgia, arthritis, fatigue	NA	NA	
8*	PGS, purpura, low C4, M-protein, cryoglobulins	Arthralgia, fatigue, Raynaud's phenomenon, vasculitis	Low C4, M-protein, cryoglobulins	none	SS
9	-	Arthralgia, fatigue		none	
10*	PGS, cryoglobulins	Fatigue, vasculitis, pulmonary, hepatic and renal involvement	cryoglobulins	pulmonary, hepatic and renal involvement	MALT-lymphoma & SS
11	PGS	None	-	none	
12	PGS	Arthralgia, fatigue	-	none	
13*	PGS, low C4,M-protein	Arthritis, fatigue, Raynaud's phenomenon, vasculitis and esophageal involvement	Low C4, M-protein	Raynaud's phenomenon, esophageal involvement	MALT-lymphoma & SS
14*	PGS, M-protein	Arthritis, fatigue, Raynaud's phenomenon	M-protein	Raynaud's phenomenon	MALT-lymphoma
15	PGS	Fatigue	-	Fatigue	MALT-lymphoma
16	-	Fatigue	-	Fatigue	
17	M-protein, low C4	Arthralgia, Raynaud's phenomenon	Low C4	Arthralgia	
18	-	Fatigue	-	Fatigue	
19	PGS	Fatigue	-	none	
20	PGS	Fatigue	NA	NA	
21	PGS	Fatigue, Raynaud's phenomenon	-	Fatigue, Raynaud's phenomenon	MALT-lymphoma
22	Low C4	Arthralgia, fatigue	-	Arthralgia	
23	PGS, normal C4	Fatigue	-	Fatigue	MALT-lymphoma
24	PGS	Arthralgia	NA	NA	
25	PGS	Arthralgia, fatigue	NA	NA	
26	PGS	Fatigue	-	Fatigue	
27	PGS	Fatigue, Raynaud's phenomenon	NA	NA	
28*	PGS, low C4, M-protein, cryoglobulins	Fatigue, vasculitis, pulmonary and esophageal involvement, polyneuropathy	Low C4	Polyneuropathy, pulmonary and esophageal involvement,	
29*	PGS, low C4, M-protein, cryoglobulins	Arthritis, Raynaud's phenomenon	Low C4, M-protein	none	MALT-lymphoma
30	PGS	Arthralgia	NA	NA	MALT-lymphoma
31			-	none	
32	PGS, low C4,	Fatigue	Low C4	none	
33	PGS, low C4		NA	NA	
34	PGS	Arthralgia		Arthralgia	
35	PGS			none	

Abbreviatons: UPN: unique patient number; *patient with high SS disease activity; see Patients and Methods; PGS: parotid gland swelling; M-protein: monoclonal protein present; NA none applicable

Table 6. Patients failing initial policy resulting in progression or recurrence of MALT-lymphoma and/or SS disease activity

Initial policy	N	MALT Stage	Initial SS disase activity	Initial treatment response	Progression/ Recurrence	Months after initial diagnosis	[Re-]treatment	Response
Watchful waiting	2	L	high	SD	MALT-lymphoma	81	Surgery	CR
		L	low	SD	MALT-lymphoma	34	Radiotherapy	CR
Surgery	2	L	low	CR	MALT-lymphoma	85	RCP	CR
		LD	low	SD	MALT-lymphoma	73	Radiotherapy	CR
Radiotherapy	1	LD	high	SD	MALT-lymphoma & SS disease activity	98	Cyclophosphamide	Stable
Surgery/Radio-therapy	1	DD	low	CR	MALT-lymphoma	39	RCP	Stable
Rituximab	4	L	high	SD	MALT-lymphoma & SS disease activity	4	Rituximab	Stable
		LD	high	CR	MALT-lymphoma	52	Rituximab	Stable
		LD	high	CR	SS disease activity	27	Cyclophosphamide	Stable
		L	low	PR	MALT-lymphoma	9	Rituximab	Stable
RCP	1	DD	high	CR	MALT-lymphoma	15	Radiotherapy	CR

Abbreviations: L, localized disease; LD, locally disseminated disease; DD, disseminated disease; SD, stable disease; PR, partial response; CR, complete response; R-CP, Rituximab with cyclophosphamide and prednisone

Figure 1: Progression-free survival of MALT-lymphoma.

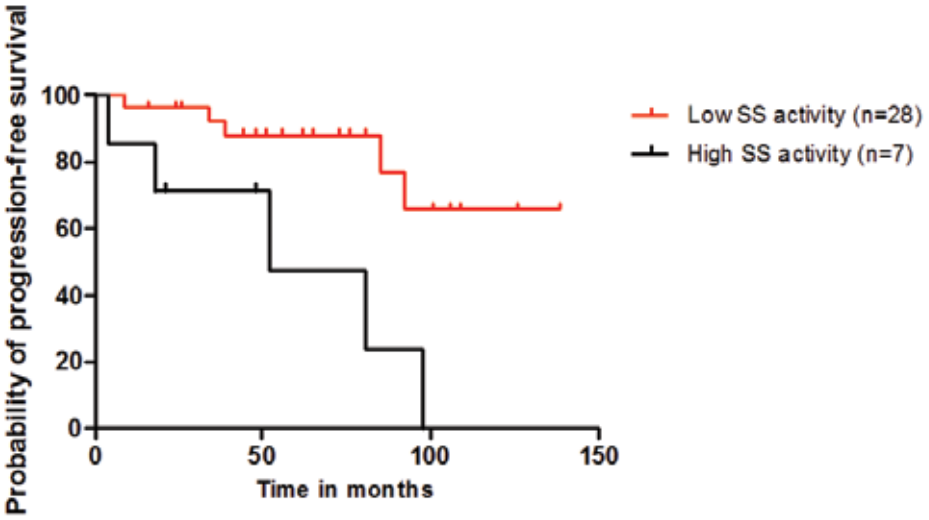

Figure 1. Progression-free survival of MALT lymphoma according to initial SS disease activity; Red, 28 MALT-SS patients with low initial SS disease activity; Black, 7 MALT-SS patients with high initial SS disease activity The difference in disease-free-survival of patients with initial low versus high SS disease activity was significant ($p < 0.05$) *One patient with high initial SS disease activity was retreated with cyclophosphamide because of SS disease activity without signs of MALT-lymphoma (table 6)

A reduction in extraglandular manifestations (arthritis, vasculitis, pulmonary, hepatic or renal SS involvement) was seen in all systemic treated patients (rituximab or R-CP). Improvement in serologic parameters (increase C4, no presence of cryoglobulins and M-protein) was observed in the majority of patients (9 out of 13) treated with rituximab. Three of the four patients that did not show improvement in serologic parameters after rituximab treatment had high SS disease activity initially. All six patients treated with R-CP showed normalization of these serologic parameters after treatment (table 5).

Long-term outcome and follow-up

After a median follow-up of 76 months (range16-153) after initial MALT-SS diagnosis, progression or recurrence of MALT lymphoma was observed in 10 out of 35 patients at a median time of 45 months after diagnosis (range 4-98 months, table 6). Five of the 10 patients with progression or recurrence of lymphoma had high SS disease activity initially (table 3). The difference in disease-free-survival of patients with initial low versus high SS disease activity was significant ($p < 0.05$, figure 1).

In the "watchful waiting" group, two of the 10 patients showed MALT-lymphoma progression after 34 and 81 months. One of these patients also had high SS disease activity at the initial MALT diagnosis. All other patients in the "watchful waiting" group had low SS disease activity.

Figure 2: Management of MALT-SS

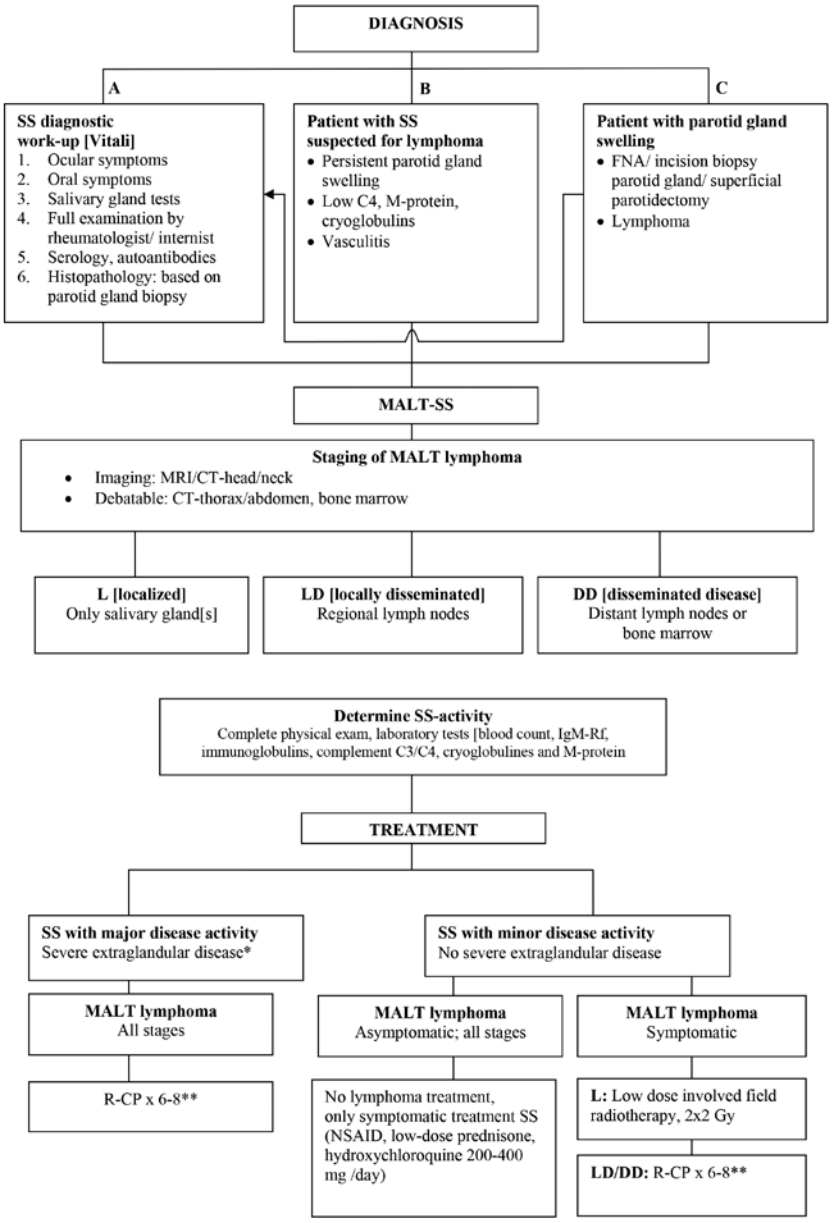

Figure 2. Clinical algorithm leading to MALT-SS diagnosis and treatment. A: patients in whom MALT-SS is diagnosed by chance at routine diagnostic biopsy for SS diagnosis; B: patients with known SS and strong clinical suspicion of lymphoma; C: patients with unknown parotid gland swelling; MALT/SS: patients with MALT lymphoma and associated Sjögren's syndrome (SS); FNA: fine needle aspiration; *extraglandular disease: polyarthritis/myositis, glomerulonephritis, nervous system involvement, cryoglobulinemic vasculitis, other severe organ involvement, serological abnormalities: cryoglobulinaemia, C4 < 0.10 g/l; **R-CP: six intravenous infusions of 375 mg/m^2 of rituximab and six-eight cycles of CP, given every 3 weeks[59].

Increased extraglandular SS activity without progression of MALT lymphoma necessitating immunosuppressive retreatment was observed in 1 patient treated with rituximab at 27 months after MALT diagnosis (table 6).
Transformation to high-grade lymphoma was not observed. Thirty-four patients are alive at a median follow-up of 76 months (range 16-153 months), one patient died of pneumonia unrelated to MALT lymphoma.

Discussion

In this retrospective study we analyzed the clinical course of patients with SS and associated MALT lymphoma. SS patients with MALT lymphoma of the parotid gland usually had localized disease. High SS disease activity at presentation, i.e. multiple extraglandular SS manifestations, low C4, presence of cryoglobulins and/ or M-protein, was associated with clinical progression or recurrence of MALT lymphoma and/or deterioration of SS during follow-up necessitating (re)treatment. Although the median follow-up of 6 years is still relatively short, transformation to high-grade lymphoma was not observed and no patient died from lymphoma.

MALT lymphoma in patients with SS is part of a spectrum ranging from indolent asymptomatic lymphoma and low SS disease activity to locally disseminated or disseminated lymphoma and severe extraglandular SS manifestations. According to the latest diagnostic consensus criteria, pre-existent lymphoma is considered to be an exclusion criterion of SS, because lymphoma of the parotid gland itself can cause mouth dryness and parotid gland swelling [42]. In our opinion, however, MALT lymphoma of the salivary gland should not be considered as an exclusion criterion for the diagnosis of SS. As also shown in the study by Ekström et al [6], the great majority of these lymphomas are associated with SS or other autoimmune diseases [17;55]. According to that study, vasculitis, peripheral nerve involvement, glomerulonephritis, fever, anaemia, and lymphopenia are observed significantly more often in MALT-SS patients than in the general SS population [5]. In our study the majority of patients (71%) had no severe extraglandular manifestations. This discrepancy might be explained by the inclusion of 11 patients in whom a MALT-lymphoma was detected during diagnostic work-up for SS, which included a parotid biopsy in our institution instead of a labial biopsy.

The parotid biopsy is preferred because of its association with less morbidity and comparable diagnostic potential to that of a labial biopsy in the diagnosis of SS [43]. Other studies have found an incidence of 3.4 to 7% of MALT-lymphoma within their SS cohort [1;2;5]. Since the parotid is the gland most commonly involved in MALT-lymphoma, the routine use of the parotid biopsy for the diagnosis of SS likely explains a slightly higher incidence of MALT-lymphoma in our SS cohort (11%) [56] as well as the higher frequency of lymphoma patients without severe extraglandular manifestations of SS.

The high survival rate in our cohort (97%, one patient died of pneumonia unrelated to MALT-lymphoma) is in accordance with literature of MALT-lymphoma not associated with SS [13;14]. Progression/relapse was seen in 29% of our patients, this finding is also in accordance with the 30% progression/relapse rate reported for MALT lymphoma not associated with SS [57].

The staging system for MALT-SS used in this study [see Patients and Methods] may provide better prognostic information at diagnosis than the traditional Ann Arbor staging. Although MALT-lymphoma in SS can localize in other mucosal sites, it usually localizes in the main target of the autoimmune disease, i.e the parotid gland, [16;56] It is debatable whether it is necessary to perform full staging in patients with MALT-SS, including CT-scans of thorax and abdomen and bone marrow biopsy. Bone marrow involvement is rare in the patients described, and probably does not influence prognosis nor treatment [12].

In some patients with locally disseminated or disseminated disease, it may be difficult to decide whether symptoms should be attributed to lymphoma activity or to SS activity. For example, B symptoms such as weight loss, might be attributed to lymphoma activity, but they could also be part of SS disease activity. In these patients, both lymphoma and SS disease activity need to be addressed: not only the clinical characteristics of the lymphoma, but also the severity of SS manifestations might determine the choice of treatment. We are aware of the fact that the use of an international standardised activity score is needed for evaluating SS disease activity. However, in the time frame this cohort was diagnosed, no standardised activity score was available [51;52]. By that time the disease activity was assessed by a team of experts according to our own standardized methods (Methods section).

As observed in this analysis, "watchful waiting" seems a suitable option in patients with asymptomatic MALT lymphoma in the absence of high SS disease activity, since most patients remained asymptomatic for a long period of time (figure 1). In patients with symptomatic MALT lymphoma, such as a persistent disabling parotid gland swelling, but with low SS disease activity, local treatment with low-dose involved field radiotherapy to spare remaining salivary function (2x2 or 1x4 Gy) might be sufficient. However, experience with low-dose involved field radiotherapy in extra-nodal MALT-lymphoma is limited.

Our study also suggests that in SS-MALT patients with initial high SS disease activity rituximab monotherapy might not be sufficient because these patients required retreatment due to recurrence of MALT-lymphoma and/or development of SS disease activity. Also, normalization of serological parameters was not seen (low C4 levels and presence of cryoglobulins and/or M-protein). In these patients treatment might have to include more intensive immunosuppressive therapy, for instance a combination of rituximab with cyclophosphamide and prednisone (R-CP). This combination therapy is effective in the treatment of both indolent lymphoma and autoimmune disease [58;59].

Current guidelines for management and treatment of patients with MALT-SS in our center are based on the treatment experience stated in this article (figure 2):

i. Asymptomatic MALT and low SS disease activity: watchful waiting
ii. Symptomatic local MALT, no- or low SS disease activity: radiotherapy;
iii. High SS disease activity and asymptomatic MALT: rituximab only (phase II trial) or immunochemotherapy: Rituximab-Cyclophosphamide-Prednisone (R-CP)
iv. Symptomatic MALT and high SS disease activity: R-CP

Although our study indicates that most treated patients fare well, recurrences may occur. Whether these patients might benefit from maintenance B-cell depletion therapy, as has been shown in indolent B-cell lymphoma and autoimmune diseases like RA and SLE, remains to be determined.

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CHAPTER 7

CHAPTER 8

Summary and General discussion

Summary

Sjögren's syndrome (SS) is a systemic autoimmune disease characterized by lymphocytic infiltrates in the exocrine glands, affecting predominantly the salivary and lacrimal glands. Patients mainly suffer from dry mouth (xerostomia) and dry eyes (keratoconjunctivitis sicca). Multiple manifestations such as fatigue, arthralgia and myalgia are frequently present and together with the dryness related complaints lead to a diminished quality of life in patients with SS. Two forms of SS exist, namely, primary SS (pSS), when the salivary and lacrimal glands are involved in the disease process, and secondary SS (sSS), when the patients also suffer from another autoimmune disease such as rheumatoid arthritis and systemic lupus erythematosus. SS can be accompanied by a lymphoid malignancy. Studies have determined the risk of developing mucosa associated lymphoid tissue (MALT) lymphoma to be between six [1] and 20 [2] times greater than within the general population. Approximately 7.5% of patients develop MALT lymphoma, particularly in the primary presentation of the syndrome [3, 4].

No acknowledged treatment for pSS exists, but B cell depletion by rituximab, a monoclonal directed against CD20 on B cells, has shown promising clinical beneficial results in several trials. In these trials most attention was given to clinical parameters, disease activity and quality of life. Less focus was given to the immunopathology in pSS during B cell depletion treatment. Information gathered on the local and systemic effects before and after B cell depletion therapy can provide insight into the immunopathogenic mechanism of pSS. In the research described in this thesis, the focus has been put on systemic immunopathological effects of B cell targeted anti-CD20 monoclonal antibody therapy (rituximab) in pSS, in particular the effects of rituximab treatment on the cytokine and chemokine profile, and on the local inflammation and regenerative potential of the epithelial component of secretory tissue of parotid glands. Furthermore, guidelines for the management of MALT lymphoma were developed in which rituximab treatment also plays a role.

There is evidence that genes and their protein products are organized into functional modules according to cellular processes and pathways. Systems biology analysis, based on microarray data, is increasingly used to provide relative measurements of mRNA levels for thousands of genes in a biological sample. In **chapter 2** a systems biology analysis of expression data from human salivary gland tissue (parotid gland) and mouse salivary gland tissue is described. This analysis demonstrated 4 significant upregulated and 3 downregulated co-expression modules in parotid glands from human pSS patients compared to control parotid glands. From these 7 co-expression modules, one human disease related module was highly preserved in the SS mouse model suggesting a shared pathway. This module was enriched with genes involved in immune and inflammatory response. These genes encode for molecules involved in

lymphocyte recruitment and molecules involved in germinal center (GC) formation. Cell type abundance assessments of the parotid gland of pSS patients compared to controls showed a predominance of B cells over other immune cells such as T cells and dendritic cells. These observations support the notion that B cells and B cell related factors, such as GCs and certain cytokines/chemokines (a.o., BAFF, IFN-α and IL-6) play an important role in human pSS pathogenesis and prompted us to study the immunoregulatory aspect, with focus on B cells and B cell related factors, in the subsequent chapters.

Based on the significance of the immune factors, and the notion that B cells predominate in the parotid glands (**chapter 2**), it was plausible to study the local effects of rituximab on the local inflammation and regenerative potential of salivary tissue after B cell targeted therapy in parotid glands. Therefore, in **chapter 3** a double blind, randomized, placebo controlled trial with rituximab in patients with pSS is described in which the local inflammation and regenerative potential of the parotid gland was assessed. Thirty pSS patients had been included in this trial of which 20 patients received rituximab and 10 received placebo treatment. In all rituximab treated patients a major reduction of the lymphocytic infiltrate and of the B/T-cell ratio was observed, while no reduction in infiltrate and B/T-cell ratio was observed in placebo-treated patients. The relative number of lymphoepithelial lesions and GC-like structures was significantly reduced in rituximab treated, but not in placebo-treated patients. In the rituximab treated patients there was no significant change in stimulated parotid salivary flow compared to baseline, while parotid flow had significantly decreased in the placebo treated patients. Thus rituximab treatment leads to major reduction of lymphocytic infiltration, GC-like structures, lymphoepithelial lesions and relative B cell component. Together with this reduction, a major loss of GC-like structures and redifferentiation of lymphoepithelial lesions into regular striated ducts was observed, reflecting major reduction of histologically verified disease activity. This reduction of inflammation in the parotid gland is reflected by a preservation of the parotid salivary flow.

Clinical effects of rituximab monotherapy have been shown to last for 6-9 months. This notion, together with the results of **chapters 2** and **3**, suggests that there are additional factors enabling the survival and reappearance of (autoreactive) B cells. B cell activating factor (BAFF) and a proliferation-inducing ligand (APRIL) are known B cell survival factors. Adding together, these observations led us to determine the effect of B cell depletion by rituximab treatment on BAFF and APRIL levels in serum and localization in parotid gland tissue (**chapter 4**). Blood samples were collected from the 30 pSS patients (20 rituximab and 10 placebo; see also **chapter 3**), at various time points. In addition, blood of 10 healthy controls was collected. Finally, for means

of localization of BAFF, parotid gland biopsies of 5 rituximab and 5 placebo treated patients were immunohistologically stained before and after treatment. The results showed increased levels of BAFF and APRIL in blood of pSS patients at baseline when compared to healthy controls. Notably, rituximab treatment resulted in a period of nearly depleted peripheral B cells and a profound increase in BAFF levels. The APRIL levels did not change after treatment. Localization assessment of BAFF in parotid glands of rituximab treated patients revealed staining of BAFF in the lymphocytic infiltrate surrounding the ducts. Despite the reduction in size of these infiltrates, BAFF staining was still clearly present. From this study it was concluded that rituximab treatment leads to a significant increase of BAFF. This increase, together with the local presence of BAFF, might explain survival of (auto-reactive) B cells and reoccurrence of SS disease activity.

The results of the study described in **chapter 2** pointed towards the critical role of cytokines and chemokines in the pathogenesis of pSS. In **chapter 4** we have shown that B cell depletion results in a rise in BAFF levels which could lead to a BAFF modulated repopulation of B cells in the blood and salivary glands of rituximab treated patients. Whether the (elevated) levels of other (pro-inflammatory) cytokine levels are affected by rituximab treatment was addressed in **chapter 5**. Blood samples at various time points from treated pSS patients (20 rituximab, 10 placebo) and blood from 10 healthy controls were analyzed for a large panel pro-inflammatory and other cytokines. The results of this study showed elevated levels for nearly all cytokines in pSS patients compared to healthy controls at baseline, reflecting the immune activity of this autoimmune disease. More importantly, when compared to placebo, a decrease in a subset of mainly pro-inflammatory cytokines (GM-CSF, IL-1Ra, IL-6, IL-10, IFN-α, TNF-α) and chemokines (MIP-1β (CCL4), MIG (CXCL9)) was observed in rituximab treated patients. After this initial decline, levels reached baseline values by 36 weeks after rituximab treatment. This study demonstrated that depletion of B cells resulted in a concomitant decrease in serum levels of a subset of mainly pro-inflammatory cytokines. These results suggest that the beneficial effect of rituximab treatment in pSS patients is mediated, at least in part, by depletion of pro-inflammatory cytokine producing B cells.

Besides the immediate effect caused by depletion of (autoreactive) B cells, rituximab treatment may also influence the frequency and function of regulatory T cells (Tregs). Tregs are a subset of T cells that play an essential role in immune homeostasis by suppression of the proliferation and function of, amongst others, effector T cells (Teffs). In **chapter 6** a study is described in which the effect of rituximab treatment on the frequency and function of Tregs was assessed. From the 30 patients of the clinical trial, blood samples at various time points were analyzed for frequency of Tregs. In addition,

blood from 10 healthy controls was analyzed. Finally, from 5 patients treated with rituximab the function of Tregs was assessed by co-culture assay at baseline and 24 weeks after rituximab infusion. The results of this study showed no significant difference between Tregs of healthy controls and pSS patients as well as that rituximab treatment did not change the frequency of Tregs. Functional analysis of Tregs revealed no change in function before and after rituximab treatment. Thus, the effectiveness of rituximab treatment in pSS patients is not associated with enhancement of peripheral Tregs numbers and/or function.

A variety of immune factors (a.o., GCs, IL-6, BAFF), as assessed in the previous chapters, is thought to play a role in the lymphoproliferation that may progress into MALT-lymphoma. In **chapter 7** a retrospective study is described on basis of which results guidelines for the management of MALT-lymphoma patients with associated SS were developed. A total of 329 SS patients was evaluated amongst which 35 MALT-pSS patients were identified with a median follow-up of 76 months. In all cases, the MALT-lymphoma was localized in the parotid gland. Treatment consisted of "watchful waiting", surgery, radiotherapy, surgery combined with radiotherapy, rituximab only or rituximab combined with chemotherapy. It was shown that MALT-lymphoma progressed in 6 out of 7 patients with initial high pSS disease activity and in only 1 out of 28 patients with initial low pSS disease activity. From this study it was concluded that an initially high pSS disease activity likely constitutes an adverse prognostic factor for progression of lymphoma. Furthermore, in pSS patients with localized asymptomatic MALT lymphoma and low pSS disease activity, a "watchful waiting" strategy seems justified. Guidelines for the treatment of pSS derived MALT-lymphoma are given.

General discussion

Several factors, including genetic predisposition and environmental triggers, have been posed to be involved in the development of SS. Although the exact primary event in the etiology of SS remains elusive, the studies described in this thesis demonstrate a critical role of B cells and B cell related immune factors in the pathogenesis of pSS.

B cells in the lymphocytic infiltrate of the salivary glands

The hallmark in the development of pSS is the formation of lymphocytic infiltrates in exocrine glands. The parotid gland tissue used in the studies described in this thesis provide the unique opportunity for repeated biopies of the same gland to study the effect in the same target organ before and after targeted therapy. In **chapter 3** it is shown that immunostaining results of parotid gland tissue of pSS patients supported the conclusion drawn from the study described in **chapter 2** that B cells predominate

in parotid gland tissue by demonstrating a predominance of B cells over T cells at baseline.

The predominance of B cells in the lymphocytic infiltrate in parotid gland tissue is in contrary to the T cell predominance in human minor labial glands. In the latter glands, T cells account for approximately 80% of the total infiltrate, with the remaining 20% composed of B cells and plasma cells [5]. It has been suggested that during the progression of SS the initial predominance of T cells is gradually reduced by an accumulation of B cells and plasma cells [6, 7]. The initial discrepancy in B cell numbers between minor salivary and parotid glands in pSS, might be caused by the more favorable niche for B cell development in the parotid gland in which gland type GC-like structures are commonly observed.

Immune factors involved in B cell development and survival

In GC-like structures, which are ectopic lymphoid microstructures, mature B cells rapidly proliferate, differentiate, mutate (through somatic hypermutation) and class switch their antibodies during an immune response. B cells become activated and undergo, presumably in the GC-like structures, antigen-driven changes such as Ig heavy chain class switching and affinity maturation. These antigen-driven changes are thought to ultimately lead to the generation of memory B and antibody-producing plasma cells [8]. It has been suggested that GC-like structures affect disease expression in pSS, exemplified by studies showing that presence of GC-like structures coincides with elevated titres of rheumatoid factor, autoantibodies, increased IgG levels and larger lymphocytic infiltrates [9, 10].

The effect of GC-like structures is mediated by the higher lymphocytic organization leading to increased disruption of glandular tissue [11]. GC-like structure formation can increase the effectiveness of communication between lymphocytes, thereby aiding in lymphocyte function [10]. Furthermore, in a retrospective study it has been proposed that the presence of GC-like structures in labial salivary glands of pSS patients might be used as a highly predictive and easy to obtain marker for lymphoma development [12].

B cell development is dependent on survival factors such as B cell activating factor (BAFF) and a proliferation induced ligand (APRIL) which were shown to be elevated in serum of pSS patients (**chapter 4**). After rituximab treatment serum BAFF level increased profoundly and immunostaining showed presence of BAFF in parotid gland tissue (**chapter 4**). In particular, survival of B cells in parotid gland tissue after rituximab therapy might be modulated by BAFF through its effects on their lifespan [13]. As a result, BAFF can act as a survival signal for B cells and may diminish the efficacy of rituximab treatment [13, 14]. Therefore, adding an anti-BAFF treatment to a treatment with rituximab has been proposed to prolong the period of remission after rituximab infusion [15].

Autoreactive B cell selection and activation after rituximab treatment

The incomplete resolving of GC-like structures and, more importantly, the observed increase of serum BAFF levels after rituximab treatment may result in undesired side effects on selection and survival of autoreactive B cells and on B cell activation. B cell receptor and BAFF-receptor (BAFF-R) signaling synergize in B cell survival, which is mediated by activation of NF-kB [16]. B cell receptor signaling activates the classical NF-kB pathway that results in the production of p100 substrate, required for BAFF-R signaling via the alternative NF-kB pathway [17]. Evidence from studies with transgenic mice indicates that BAFF levels regulate the stringency of the selection of naive B cells [13, 18, 19]. Current models suggest that naive B cells expressing a transgenic surface Ig that binds DNA or protein autoantigens first attempt to alter the B cell receptor by further variable gene rearrangement using "receptor editing" [20, 21]. If receptor editing is unsuccessful, then the offending B cell may be eliminated by clonal deletion [22] or it may enter maturity, although with reduced or altered function so that it no longer reacts to the self-antigens, which is referred to as clonal anergy [23, 24].

The half-life of anergic B cells, when compared to non-anergic B cells, is substantially decreased when BAFF levels are reduced [18]. Excess of BAFF rescues autoreactive cells that are anergized after the early transitional B cell stage, but not at the early B cell stage [18]. One possible explanation, for the poor survival response when BAFF levels are limited, is that autoreactive transitional B cells down regulate their B cell receptors, which results in less availability of substrate (p100), required for BAFF-R signaling. As a consequence these cells require higher BAFF levels for their survival and differentiation to mature B cells [25]. In contrast, long-lived memory B cells that arise after an antigen driven autoimmune response, appear to be completely independent from BAFF (and APRIL) [25, 26]. It might therefore be that the increased peripheral expression and local presence of BAFF could lead to survival of the (auto-) reactive B cell in the GC-like structures. The majority of re-emerging B cells after rituximab treatment are transitional type B cells [27] and it remains to be seen that this B cell fraction comprises more autoreactive cells compared to placebo treated patients.

The notion that BAFF levels regulate the selection of naïve B cells is based on studies with (genetically-engineered) mice (reviewed in Liu and Davidson [28]). Studies in human are lacking. Whether the elevated serum BAFF levels observed after rituximab treatment also impair the stringency of the selection of naive and/or memory B cells in pSS patients, and therewith promote autoreactivity, is not known. In conclusion, the possibility that the persistence of GC-like structures and elevated BAFF levels contribute to the new formation of autoreactive cells from the bone marrow and lymphoproliferation cannot be completely ruled out, but, probably BAFF alone does not affect the survival of long-lived autoreactive memory B cells.

Role of IL-6 in development and survival of B cells

At the B cell level, IL-6 stimulates B cell proliferation and differentiation to plasma cells, amongst others via IL-21, which contribute to the B cell hyperreactivity and hypergammaglobulinemia seen in patients with pSS [29]. More importantly, IL-6 stimulates GC-like structure formation, amongst others, by CXCL13 and IL-6 induced production of IL-21 [30-32].

In pSS patients, it was shown that IL-6 serum cytokine levels are increased at baseline and decrease after rituximab treatment (**chapter 5**). The decrease of IL-6 after rituximab treatment is in conjunction with a decrease in GC-like structures (chapter 3), which suggests a linked pathway. Furthermore, IL-6 has been shown to play an important role in immunopathology by the generation of Th17 pro-inflammatory lymphocytes [33], which lymphocytes seem to be involved in the formation of GC-like structures in the salivary glands [34]. Thus, reduced levels of IL-6, after rituximab treatment, may result in fewer Th17 cells. In rheumatoid arthritis, a reduction in the Th17 response in the synovial tissues of patients has been observed after rituximab therapy too [35]. The observed decrease of IL-6 (**chapter 5**), after rituximab treatment, could explain the reduction in GC-like structures (**chapter 3**) in salivary gland tissue. Reduced IL-6 levels seen after rituximab treatment may also cause less B cell activation of remaining local B cells. Furthermore, since this cytokine is also is an important survival factor for plasma cells, rituximab could also affect, indirectly via IL-6, the number of plasma cells in the body and the IgG levels seen in serum. Indeed, in vitro experiments have shown that there is a strong correlation between concentration of IL-6 and IgA or IgG secretion by plasma cells [36].

Etiology and immunoregulatory pathway

Although the findings reported in this thesis share light on the immune factors involved in the pathogenesis of pSS, the exact etiology remains obscure. A viral trigger has been implicated in the initial development of SS. It is known that most viruses can induce production of type I interferons. Interferons (IFNs) are a family of cytokines derived from different cell types in response to many stimuli including bacteria, viruses, foreign cells, macromolecules, and other chemical compounds. The majority of INFs constitute of the 13 IFN-α subtypes [37, 38].

A pathologic overproduction of type I IFN has been observed in autoimmune diseases, such as systemic lupus erythematosus, type 1 diabetes mellitus and dermatomyositis [39]. Serum IFN-α levels are found to correlate with activities and severity of both diseases as well as some clinical manifestations such as fever and skin rashes in patients with systemic lupus erythematosus [40, 41]. Recently, overproduction of type I IFN has been noted in pSS patients too [39, 42]. IFN production was observed in both in sera (**chapter 5**) and parotid salivary gland biopsy specimens (**chapter 2**) of the latter patients.

Type I IFN, which can be produced by, amongst others, plasmacytoid dendritic cells and epithelial cells, has strong antiviral effects but can also enhance proliferation of autoreactive B cells and the autoantibody production [43, 44]. Type I IFN can also increase the expression and exposure of autoantigens to autoreactive B cells and T cells [45, 46], the underlying mechanism is yet unknown. More interestingly, BAFF expression is largely increased by stimulation with type 1 or 2 IFN [47].

BAFF is an IFN-induced gene and studies have shown that BAFF-expression is through a type I IFN-dependent mechanism [48]. Previous studies have shown that TNF blockers are not effective in the treatment of pSS. It was shown that treatment of pSS patients with TNF blocker etanercept led to a significant increase in plasma IFN-α [49]. The increase of IFN-α leads to an increase in BAFF. This indicates BAFF as a more relevant therapeutic target for the disease [49].

It can be speculated that viral infection, in genetic and hormonal predisposed individuals, results in activation and overproduction of the type I IFN system in the salivary glands (**chapter 2**) which may be involved in the development of pSS. Virus-specific T lymphocytes may lead to (over-)production of type II IFN-γ (**chapter 5**). IFN type I and II would drive the inflammation leading to the formation of neoepitopes and breakdown of self tolerance, achieved in part by the oxidative damage of certain ribonucleoprotein particles, such as proteins Ro and La, as observed in systemic lupus erythematosus [50], Activated B cells would become autoreactive memory B cells in GC-like structures (**chapter 3**), under stimulation of BAFF (**chapter 4**). and stimulate the production of proinflammatory cytokines, such as IL-6 and TNF-α (**chapter 5**). These pro-inflammatory cytokines lead to the perpetuation of GC-like structures, inhibition of Tregs (**chapter 6**) [51] and continuous tissue inflammation. Continuous tissue inflammation results in tissue destruction, decreased glandular function, extraglandular manifestations and increased risk of MALT-lymphoma development (**chapter 7**). Indeed we have demonstrated that a decrease of B cells could lead to a decrease in IFN type I/II (**chapter 5**) and may account, in part, for the beneficial effects of rituximab treatment.

Future perspectives

In this thesis it is shown that monotherapy of rituximab, although beneficial in improving extraglandular manifestations, might not be sufficient to improve parotid glandular function and maintain clinical beneficial effects longer than 36 weeks in pSS patients. It is also shown that local B cells and plasma cells [52] survive after rituximab treatment in pSS, probably because of a survival niche mediated by BAFF, and that rituximab treatment leads to a profound elevation of peripheral BAFF levels. These observations may have implications for future treatment regiments in pSS.

It can be speculated that anti-BAFF or anti-IL-6 therapy, e.g., belumimab and tocilizumab respectively, may be beneficial in the treatment of pSS patients as

complementary therapy aside B cell depletion with rituximab. Indeed clinical trials with anti BAFF and anti IL-6, although not combined with rituximab, have already proven successful in rheumatoid arthritis [53] and systemic lupus erythematosus [54, 55]. Effects of these treatment regiments are currently unknown in pSS and clinical trials are eagerly awaited [56]. Currently, at our department, anti BAFF and anti IL-6 trials are designed to assess the effect on clinical and immunohistopathology in pSS. Furthermore, combining the targeting of BAFF with rituximab treatment could enhance and prolong the effect of rituximab in pSS.

In addition to the crucial role of B cells exemplified in this thesis, T cells play a fundamental part in the pathogenesis too. For example, effector CD4+ T helper (Th) cells are required for humoral immune responses to the ubiquitously expressed ribonucleoproteins, the proteins the autoantibodies against Ro/SSA and La/SSB are directed to. These CD4+ Th cells are essential for the generation of plasma cells and the formation of memory cells in the GC-like structures in the salivary glands [57]. CD4+ Th cells are also critically involved in the inflammatory response in exocrine glands and their cytokines affect directly ductal epithelial cells [58]. Most of the activated CD4+ T cells in salivary glands of pSS express cytokines such as IFNγ and TNFα, which are classically considered to be prototypic for Th1 cells [59]. In addition to Th1 cells, also Th17 cells have been shown to be involved in the pathogenesis of pSS [57].

Activation of CD4+ T cells requires recognition of the T cell receptor of peptide bound to MHC class II molecules and co-stimulation by binding of the co-stimulatory molecules CD80/CD86 to CD28 expressed on the surface of the T cells [60, 61]. Dendritic cells are critically involved in the full activation of naïve CD4+ T cells. These cells not only present antigen to the T cells, but also deliver the required co-stimulatory signals [62]. In order to receive appropriate help from effector CD4+ T cells, B cells also present antigen to the T cells and express the co-stimulatory molecules CD80/86, in order to become either a memory or plasma cell [63]. Interestingly, ductal epithelial cells in salivary glands of pSS patients also appear to act as antigen presenting cells [64]. Probably under the influence of pro-inflammatory cytokines, these epithelial cells not only express MHC class II molecules, but, importantly, also express the co-stimulatory molecules CD80/86 [64]. The role of this form of antigen presentation to T cells in pathogenesis of pSS remains to be elucidated. The biological disease modifying anti-inflammatory rheumatic drug abatacept inhibits co-stimulation of T cells by binding with high affinity to the molecules CD80 and CD86, herewith blocking their binding to CD28 on the surface of T cells [65]. It is expected that in absence of appropriate co-stimulation by CD28 both arms of the adaptive immune responses will be inhibited. This inhibition may lead a.o. to reduced inflammation (by Th1 cells), decrease in ectopic lymphoid tissue formation (by Th17 cells), decreased memory B cell formation (by follicular helper T cells) and plasma cell formation (by Th1 and Th2 cells). Ultimately these inhibitory effects will result in lower disease activity in pSS patients. Abatacept is

currently used for the treatment of rheumatoid arthritis and it appears to be safe and effective [66]. First results of the open label trial with abatacept at our department reveal that abatacept treatment is well tolerated, safe and effective with respect to disease activity, laboratory parameters and general fatigue in patients with pSS.

Treatment of SS has been just symptomatic for a long time. The increasing availability of targeted treatment modalities has created possibilities for intervention in pathogenic pathways involved in the disease. This availability has also provided insight into the pathogenesis of pSS. Unlike most other diseases accompanied by lymphocytic infiltration, the salivary glands are accessible to biopsy, before and after treatment. Therefore, pSS can serve as a model for parallel autoimmune disorders of organ infiltration.

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CHAPTER 8

CHAPTER 9

Nederlandse samenvatting

In **hoofdstuk 1** wordt kort de geschiedenis van het syndroom van Sjögren (SS) beschreven en de opbouw van het proefschrift uiteengezet. SS is een systemische auto-immuunziekte die wordt gekenmerkt door lymfocytaire-infiltraten in de exocriene klieren, in het bijzonder in de speeksel- en traanklieren. De lymfocytaire infiltraten bestaan voornamelijk uit B- en T-cellen en leiden uiteindelijk tot destructie van deze klieren. Twee vormen van SS kunnen worden onderscheiden, namelijk het primaire SS (pSS), wanneer alleen de speeksel- en traanklieren zijn betrokken bij het ziekteproces, en het secundaire SS (sSS), wanneer de patiënten tevens lijden aan een andere auto-immuunziekte, zoals reumatoïde artritis of systemische lupus erythematodes.

Het merendeel van de patiënten met SS ondervindt hinder van het gevoel van een droge mond (xerostomie) en droge ogen (keratoconjunctivitis sicca). Tevens zijn vaak een groot aantal andere manifestaties aanwezig, in het bijzonder, vermoeidheid, gewrichtspijn en spierpijn. Samen met de droogheidsklachten leiden deze manifestaties tot een beperking van de dagelijkse activiteiten en een verminderde kwaliteit van leven.

Kenmerkend voor het ziekteproces is dat de B-lymfocyten hyperactief zijn. Dit uit zich ondermeer in het feit dat patiënten met pSS een te hoge hoeveelheid immuunglobulinen (antilichamen) in hun bloed hebben (hypergammaglobulinemie), alsmede antilichamen gericht tegen 'eigen' lichaamscomponenten (autoantilichamen). Ook is de samenstelling van de B cel subpopulaties in het bloed veranderd, vergeleken met gezonde individuen. In de geïnfiltreerde speekselklieren leidt deze hyperactiviteit tot aanwezigheid van kiemcentra. Dit zjjn morfologisch herkenbare structuren waar geheugen B cellen gemaakt worden. Ook kunnen er klonale expansies van B cellen aangetroffen worden. Deze klonen kunnen ontsporen waardoor er een kwaadaardige woekering van B-lymfocyten optreedt. Patiënten met SS hebben dan ook een sterk verhoogd risico op het ontwikkelen van lymfomen. Dit betreft vooral het zogenaamde mucosa-associated lymfoid tissue (MALT) lymfoom. Studies hebben aangetoond dat het risico voor een SS patiënt om een MALT-lymfoom te ontwikkelen zes tot 20 keer hoger is dan het risico hierop in de algemene populatie. Ongeveer 7,5% van de SS patiënten ontwikkelt uiteindelijk een MALT lymfoom. Vooral in patiënten met pSS is de kans op het ontwikkelen van een dergelijk lymfoom verhoogd.

Er is nog geen erkende behandeling voor SS die direct ingrijpt in het ziekteproces; het merendeel van de huidige behandelingen is gericht op het verlichten van de gevolgen van en klachten gerelateerd aan SS. Recent zijn nieuwe mogelijkheden geschapen om, door gebruik te maken van therapeutische monoklonale antilichamen, ook te interveniëren in het ziekteproces zelf om zo de ziekteactiviteit van SS te remmen en verdere progressie te voorkomen. Deze monoklonale antilichamen grijpen vaak aan op specifieke eiwitten die zich op het celmembraan van B- en T-cellen bevinden of zijn gericht tegen communicatiemoleculen (cytokinen) van het immuunsysteem. Van rituximab, een monoklonaal antilichaam gericht tegen het CD20 eiwit op B cellen, zijn

in verschillende studies klinisch gunstige resultaten aangetoond in patiënten met pSS. Behandeling met rituximab leidt tot een daling van het aantal B cellen in het perifere bloed. In de tot dusver verrichte studies met rituximab is de meeste aandacht besteed aan klinische parameters, zoals ziekteactiviteit en kwaliteit van leven. Minder aandacht is besteed aan de immunopathologie van pSS tijdens B cel depletie. Nadere bestudering van de lokale en systemische effecten van B cel depletie therapie kan inzicht geven in het onderliggende immunopathogenetische mechanisme in pSS. In het in dit proefschrift beschreven onderzoek is de nadruk gelegd op de immunopathologische effecten van B cel gerichte therapie met rituximab in pSS, in het bijzonder op de effecten van een behandeling met rituximab op het cytokinen en chemokinen profiel in het bloed en de histopathologische veranderingen in het weefsel van de glandula parotidea (de oorspeekselklier). Daarnaast is nagegaan hoe een MALT-lymfoom in pSS patiënten het beste kan worden behandeld. In deze behandeling speelt rituximab eveneens een rol.

Genen en hun eiwitproducten kunnen worden onderverdeeld in functionele modules op geleide van de betrokken cellulaire processen en routes. Door middel van analyse technieken uit de systeembiologie kan inzicht worden verkregen in deze modules. Met behulp van microarrays kunnen van duizenden genen in een weefselmonster relatieve metingen op mRNA niveau worden gedaan. In **hoofdstuk 2** worden de uitkomsten van een dergelijke analyse van humaan (gl. parotidea) en muizen (gl. submandibularis/sublingualis en gl. parotidea) speekselklierweefsel beschreven. In dit onderzoek is weefsel van de oorspeekselklier gebruikt van zowel SS patiënten als van patiënten met monddroogheidsklachten die geen SS bleken te hebben (sicca patiënten) en gezonde controles. Uit deze analyse komen een zevental co-expressie modules (ziekte-gerelateerde gen-netwerken) naar voren waarbij de expressie van genen bij pSS anders is dan bij controle individuen. Bij 4 co-expressie modules komen de genen bij pSS significant verhoogd tot expressie en 3 co-expressie modules komen de genen juist verminderd tot expressie. Eén van deze modules vertoonde zowel bij humaan SS oorspeekselklierweefsel als bij speekselklierweefsel verkregen van een muizenmodel voor SS grote overeenkomsten tussen de tot expressie gebrachte genen en hun eiwitproducten. De sterke mate van overeenkomst in deze co-expressie module suggereert een gedeelde route in de ontwikkeling van SS. Deze gemeenschappelijke co-expressie module wordt gekenmerkt door genen die zijn betrokken bij de immuun- en inflammatoire respons. De betrokken genen coderen voor moleculen, zoals chemokinen, die een rol spelen bij het mobiliseren van specifieke lymfocyten en voor moleculen betrokken bij de vorming van kiemcentra. Genen die coderen voor B cellen bleken te zijn oververtegenwoordigd ten opzichte van genen coderend voor andere imuuncellen, zoals T cellen en dendritische cellen. Deze waarnemingen steunen de hypothese dat B cellen en B cel gerelateerde factoren, zoals de vorming van kiemcentra en bepaalde cytokinen/chemokinen (o.a., BAFF, IFN-α en IL-6) een

belangrijke rol spelen in de pathogenese van pSS. De uit de systeembiologische analyse naar voren gekomen bevindingen hebben ons aangezet het immunoregulatoire aspect in pSS, met nadruk op de rol van B cellen en B cel gerelateerde factoren, nader te onderzoeken.

Gezien het belang van de B cellen bij de destructie van de speekselklieren, werden de effecten van B cel depletie door rituximab op lokale ontstekingsparameters en regeneratieve mogelijkheden van het speekselklierweefsel bestudeerd. In **hoofdstuk 3** zijn de histopathologische uitkomsten van een dubbelblinde, gerandomiseerde, placebo gecontroleerde studie met rituximab in patiënten met pSS beschreven. Biopten van de oorspeekselklier van 30 pSS patiënten zijn bestudeerd. Van deze patiënten waren 20 patiënten behandeld met rituximab en 10 met een placebo. In tegenstelling tot de patiënten die een placebo kregen, werd in alle rituximab behandelde patiënten een aanzienlijke vermindering van de lymfocytaire infiltratie en de B/T-celverhouding waargenomen. In tegenstelling tot het bloed werd geen totale depletie van B cellen waargenomen in het weefsel van de oorspeekselklier. Het relatieve aantal lymphoepitheliale laesies (een bepaald type beschadiging van klierbuisjes in de speekselklier) en kiemcentrum-achtige structuren was significant verminderd in de rituximab behandelde patiënten, maar bleef onveranderd in de patiënten die een placebo hadden gekregen. Deze tekenen van een verminderde ontstekingsactiviteit en herstel van het weefsel van de oorspeekselklier in rituximab behandelde patiënten leidden niet tot significante verandering in de secretie van gestimuleerd speeksel door de oorspeekselklier. In patiënten die met een placebo waren behandeld, was de secretie van dit type speeksel juist gedaald.

De klinische effecten van rituximab monotherapie houden, na injectie met rituximab, 6-9 maanden aan. De gerapporteerde resultaten in **hoofdstuk 3**, suggereren dat niet alle (autoreactieve) B cellen in de oorspeekselklier zijn gedepleteerd. Zowel het cytokine BAFF (B cel activerende factor) en het cytokine APRIL (een proliferatie-inducerende ligand) zijn factoren die een rol spelen in de activatie en overleving van B cellen. In **hoofdstuk 4** wordt het effect van behandeling met rituximab op de BAFF en APRIL concentraties in serum beschreven. Ook is gekeken naar de lokalisatie van BAFF in de oorspeekselklier. Voorafgaand aan de behandeling met rituximab, bleken de serum concentraties van BAFF en APRIL in het bloed van pSS patiënten verhoogd vergeleken met gezonde controles. Behandeling met rituximab resulteerde in een sterke toename van BAFF concentratie in het bloed, terwijl de APRIL concentratie onveranderd bleef. BAFF kon zowel voor als na behandeling met rituximab in biopten van de oorspeekselklier worden aangetoond, namelijk in de lymfocytaire infiltraten rond de afvoerbuizen. Een duidelijk effect van rituximab op deze BAFF expressie kon echter op het niveau van het speekselklierweefsel zelf niet worden aangetoond. Uit

deze studie werd geconcludeerd dat rituximab behandeling leidt tot een significante toename van BAFF in het bloed. Deze stijging in serum en de lokale aanwezigheid van BAFF op weefselniveau kunnen een rol spelen bij de terugkeer van B cellen in het bloed of een verklaring vormen voor de overleving van (autoreactieve) B cellen en het hernieuwd optreden van ziekteactiviteit.

De resultaten van de in **hoofdstuk 2** beschreven studie duiden ook op een cruciale rol van cytokines en chemokines in de pathogenese van pSS. Uit de resultaten van de in **hoofdstuk 4** beschreven studie komt naar voren dat B cel depletie leidt tot een stijging van BAFF concentratie in bloed en derhalve tot een BAFF gemoduleerde repopulatie van B cellen in bloed en speekselklieren van rituximab behandelde patiënten.

Op welke andere (pro-inflammatoire) cytokinen een behandeling met rituximab van invloed is, is beschreven in **hoofdstuk 5.** Vergeleken met gezonde controles, zijn in pSS patiënten de serumconcentraties van bijna alle geteste cytokines verhoogd. Deze bevinding past bij de toegenomen activiteit van het immuunsysteem in deze auto-immuunziekte. Behandeling met rituximab resulteert in een daling van niet alle cytokinen die onderzocht zijn, maar slechts een deel hiervan (8 van de 25). De cytokinen waarvan de serumconcentraties waren verlaagd, betroffen voornamelijk pro-inflammatoire cytokinen (GM-CSF, IL-1Ra, IL-6, IL-10, IFN-α, TNF-α) en chemokinen (MIP-1β (CCL4), MIG (CXCL9)). Na een aanvankelijke daling van deze cytokinen en chemokinen, werden 36 weken na een behandeling met rituximab de uitgangswaarden weer bereikt. Met andere woorden, depletie van B cellen resulteert in een gelijktijdige tijdelijke verlaging van serumspiegels van vooral pro-inflammatoire cytokines. Het waargenomen klinisch gunstige effect van rituximab bij patiënten met pSS wordt mogelijk mede gemedieerd, hetzij direct, hetzij indirect door een daling van pro-inflammatoire cytokinen producerende B cellen.

Naast een daling van het aantal (autoreactieve) B cellen, kan behandeling met rituximab ook van invloed zijn op de frequentie en de functie van regulatoire T cellen (Tregs). Bij andere auto-immuunziekten (zoals reumatoïde artritis en systemische lupus erythematodes) resulteerde rituximab behandeling in een effect op de Tregs. Tregs zijn een subset van T cellen die een belangrijke rol spelen door onderdrukking van de proliferatie en functie van onder andere effector-T-cellen (Teffs). In **hoofdstuk 6** wordt een studie beschreven waarin het effect van rituximab op de frequentie en de functie van Tregs is onderzocht. In deze studie kon voorafgaand aan de behandeling geen significant verschil worden aangetoond tussen Tregs van gezonde controles en pSS patiënten. Tevens liet de functionele analyse van Tregs geen verandering zien in zijn onderdrukkende functie vóór en na behandeling met rituximab. Tenslotte werd ook op weefselniveau geen verschil in aantal Tregs waargenomen vóór en na rituximab behandeling. Met andere woorden, de effectiviteit van rituximab bij pSS patiënten lijkt

niet samen te hangen met een verhoging of veranderde functie van perifere/lokale Tregs

Een verscheidenheid van immuunfactoren (o.a. aanwezigheid van kiemcentra, verhoogde concentratie IL-6 en BAFF) wordt verondersteld een rol te spelen in de proliferatie van lymfocyten resulterend in de vorming van een MALT-lymfoom bij pSS. Over hoe een MALT-lymfoom bij pSS patiënten moet worden behandeld bleek echter geen eenduidigheid te bestaan. In **hoofdstuk 7** wordt een retrospectieve studie beschreven op basis waarvan richtlijnen voor de behandeling van pSS patiënten met een MALT-lymfoom zijn ontwikkeld. Een groep van 329 SS patiënten werd geëvalueerd, 35 van deze patiënten leden aan MALT-pSS. In alle gevallen was het MALT-lymfoom gelokaliseerd in de oorspeekselklier. Afhankelijk van het histopathologisch beeld en een aantal klinische en serologische parameters bestaat de behandeling van MALT-pSS uit "watchful waiting", chirurgie, radiotherapie, chirurgie in combinatie met radiotherapie, alleen rituximab of rituximab in combinatie met chemotherapie.

In **hoofdstuk 8** worden de implicaties van de bevindingen uit het onderhavige promotieonderzoek besproken en perspectieven voor toekomstig onderzoek beschreven.

CHAPTER 9

Dankwoord

The journey of a thousand miles begins with a single step - Lao Tzu

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DANKWOORD

Curriculum Vitae

CURRICULUM VITAE

Rodney Pollard was born on February 19, 1980 in Rotterdam, the Netherlands. He attended primary school in Paramaribo (Surinam) and Rotterdam (the Netherlands). After finishing gymnasium (Einstein Lyceum, Rotterdam) in 1999 he started Biomedical Sciences at the University of Leiden. From 2001, he combined his training in Biomedical Sciences with Medicine, again at the University of Leiden. During his studies he worked as a student research assistant at the section Parasitology, University of Indonesia, Jakarta, Indonesia (in 2003) and performed volunteer work at the hospital Obras Sociales Del Hermano Pedro, Antigua, Guatemala (in 2005). He completed Medical school in 2006 and Biomedical Sciences in 2007. In 2008, he started his PhD project at the department of Oral and Maxillofacial Surgery of the University Medical Center Groningen. In the same year, he also started with his study Dentistry at the University of Groningen. In November 2012 he started his residency in oral and maxillofacial surgery at the University Medical Center Groningen (head: prof. dr. F.K.L. Spijkervet).

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